



Thomas Kjellström

CULTURED HUMAN SKIN FIBROBLASTS AND DIABETES MELLITUS

**An experimental study on connective tissue components
and lysosomal enzymes**



Malmö 1985

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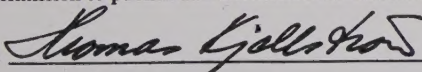


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Title and subtitle Cultured human skin fibroblasts and diabetes mellitus. An experimental study on connective tissue components and lysosomal enzymes.		
Abstract A cell culture system was elaborated for biosynthetic studies, using human skin fibroblasts from non-diabetic, type 1 and 2 diabetic subjects. Heparin, a glycosaminoglycan, was demonstrated to increase the ³H-proline incorporation into extracellular collagenase-digestible protein (collagen) by 50 per cent in the three cell types. Platelet-derived growth stimulating activity increased the production of collagen in growth-arrested non-diabetic fibroblasts with increasing concentrations of platelet supernatant, without affecting the cell number. Insulin was not affecting the intra- or extracellular activity of lysosomal enzyme, β-hexosaminidase, in cells from type 2 diabetics and non-diabetics. Glucose was, in contrast, found to increase the extracellular activity by 100 per cent in high glucose concentrations, similarly in both groups of cells. Collagen production was found to be enhanced by insulin in a dose-response relationship within physiological insulin levels. Fibroblasts from type 1 and type 1 diabetic donors appeared to be more responsive at low insulin levels. Glucose was demonstrated to increase the collagen production, as compared to basal glucose levels, in non-diabetic cells and even more in cells from type 1 diabetics. Glucose had no effect on collagen production by cells from type 2 diabetics. The production of connective tissue components and lysosomal enzyme by cultured human skin fibroblasts has been shown to be influenced by exogenous factors, and some differences have been found between cells from non-diabetic and diabetic subjects.		
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CULTURED HUMAN SKIN FIBROBLASTS AND DIABETES MELLITUS
An experimental study on connective tissue components and
lysosomal enzymes

AKADEMISK AVHANDLING

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Cultured human skin fibroblasts and diabetes mellitus.

An experimental study on connective tissue
components and lysosomal enzymes.

By

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Malmö 1985

NON VOLAT IN BUCCAS ASSA COLUMBA TUAS
(Medieval Latin)

To Eva,
Claes and Anders

LIST OF PAPERS

This thesis is based on the following papers referred to in the text by their Roman numerals.

- I. Stavenow L, Kjellström T and Malmquist J: Stimulation of collagen production in growth-arrested myocytes and fibroblasts in culture by growth factor(s) from platelets. *Exp Cell Res* 136:321-325, 1981.
- II. Kjellström T and Malmquist J: Effects of heparin and dextran sulphate on the production of collagen and protein in diabetic and non-diabetic human skin fibroblast cultures. *Med Biol* 61:186-190, 1983.
- III. Kjellström T and Malmquist J: Insulin effects on collagen and protein production in cultured human skin fibroblasts from diabetic and non-diabetic subjects. *Horm Metab Res* 16:168-171, 1984.
- IV. Kjellström T: Influence of glucose on collagen and protein production in cultured human skin fibroblasts from diabetic and non-diabetic subjects. Submitted for publication.
- V. Hultberg B, Isaksson A and Kjellström T: The effects of glucose and insulin on β -hexosaminidase in cultured fibroblasts from diabetic and non-diabetic subjects. *Ann Clin Biochem* 22, in press, 1985.

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1. ABBREVIATIONS

BTG	= β -thromboglobulin
CBM	= capillary basement membrane
CPC	= cetylpyridinium chloride
CPM	= counts per minute
DNA	= deoxyribonucleic acid
EGF	= epidermal growth factor
FBS	= foetal bovine serum
GAG	= glycosaminoglycans
HA	= hyaluronic acid
HCl	= hydrochloric acid
HEPES	= N-2-hydroxyethyl-piperazine-N'-2-ethanesulphonic acid
HSF	= human skin fibroblasts
MEM	= minimum essential medium
NEM	= N-ethylmaleimide
PBS	= phosphate buffered saline
PDGF	= platelet derived growth factor
S-GAG	= sulfated glycosaminoglycans
SMC	= smooth muscle cells
TCA	= trichloroacetic acid

2. BACKGROUND

A. Cell cultures

Cell culture offers a unique opportunity to examine environmental influence under controlled experimental conditions as well as to evaluate the genetic behaviour of cell lines. Such an in vitro model is of obvious interest in evaluating pathophysiological mechanisms in etiologically heterogeneous diseases, e.g., diabetes mellitus. Cells in culture have also gained application as a suitable model for studying acquired and inherited metabolic disorders in vitro.

The beginning of tissue culture dates back to the work of Ross Harrison in 1907 (1). Cell culture was pioneered by the group headed by Wilton Earle (2). They were the first to grow cells direct on glass in large numbers, the first to grow cultures from single cells, and the first to propagate cells intentionally in suspension (3). Through the development of synthetic media around 1950, and the subsequent development of sterile materials, cell culturing has developed rapidly in many fields (3).

B. In vitro life span and genetic expression in cultured fibroblasts

The use of cell culture in exploring heterogeneous states such as diabetes mellitus is based upon the assumption that the cultured cell has inherent mechanisms for replication and metabolism, remaining essentially unchanged and consistent several generations after removal from the in vivo environment. Hayflick (4) was the first to demonstrate a finite life span of human embryonic and adult diploid cell strains, and also an apparent inverse relation between the in vitro fibroblast life span and the chronological age of the donor (5). Martin et al. (6) demonstrated a linear decrease in the in vitro life span of fibroblasts as a function of donor age with a reduction of two cell doublings per decade. The

average growth potential of approximately 44 cell doublings from foetal skin was reduced to a mean of less than 30 cell doublings in cells from 70-80 year-old donors. They emphasized the importance of standardizing the site for the biopsy because the replicative life span of the cultures was also found to be a function of the tissue of origin. These results were confirmed by Schneider et al. (7). Interestingly enough, an in vitro decrease has also been demonstrated by Bierman (8) for the number of population doublings of human arterial smooth muscle cells as a function of donor age. Below the donor age of 20, cumulative cell population doublings (9.2) were significantly greater than those obtained from donors aged 50 or more (5.5). Cells from older donors also had longer latent periods for outgrowth from explants, as did fibroblasts, but no consistent differences were observed in population doublings or outgrowth latency among cell strains obtained from different sites along the arterial tree.

Studies on the replicative life spans of cultured human skin fibroblasts have also been extended to diabetic donors. The data from these observations have shown that cellular defects in diabetes mellitus can be expressed in vitro as a decreased growth potential in cells of extrapancreatic origin (9). The results of Goldstein et al. (9) indicated that both clinically apparent diabetics and subjects genetically predisposed (both parents had maturity-onset diabetes, type 2) to diabetes showed the inverse correlation between donor age and replicative life span of cultured fibroblasts, whereas carefully selected normal individuals failed to demonstrate this phenomenon. The discrepancy in normal cells from the studies above (5, 6, 7) was attributed to variances in donor subjects. Goldstein et al. claimed that in the earlier studies most tissue donors had been randomly chosen from living subjects and from subjects at post mortem, many of them with overt pathology. However, in a second comment (10) the authors had to correct some numerical and biostatistical errors in the original

report and the revised results reduced the discrepancy discussed. It also provided a testing of the hypothesis that the fibroblast life spans for diabetics < "prediabetics" < normals, indicating that the fibroblast replicative life span did decline with increasing predisposition to diabetes ($p = 0.04$). These authors (11) have therefore concluded that a persistent, heritable abnormality is present in cells from mesenchymal tissue of donors with overt diabetes or the predisposition to this disease. They also stated that no significant intra- or intergroup differences related to the sex of the donors were found that would influence the results reported. This is a most important note since in many mammalian species, including man, females have a longer mean survival than males, and this difference is assumed to be genetic.

There is also evidence for a relation between longevity of mammalian species and in vitro doubling potential of fibroblasts (12, 13). The absence of intersex differences in the longevity of human skin fibroblast cultures is confirmed by Ebbesen (14). He could not demonstrate any intersexual differences in plating efficiency (per cent cells attached 24 h after seeding) nor in cloning efficiency (160 cells distributed to microtiter wells).

Plating efficiency has been demonstrated by Goldstein et al (15) to be diminished in cultured human skin fibroblasts from a group of healthy individuals, who were the offspring of two clinically overt diabetics. The group was clinically normal, with fasting euglycemia, but included subjects with abnormal glucose tolerance test(s). The life spans of control and "high-risk" cells were statistically indistinguishable. The plating efficiency was 12% for the control cells and 7% for the "high-risk" cells, which is a highly significant difference. The difference was supposed to be due to an effect of the diabetic gene(s) to decrease the growth capacity of some or all fibroblasts.

Although the concept of a restricted replicative life span of cultured fibroblasts from idiopathic diabetics (not occurring in



hyperglycemia due to pancreatitis) has been further confirmed (16), it has also been modified (17) and denied (18). Vracko and McFarland (17) studied the cumulative replicative life span of skin fibroblasts from 11 diabetic donors (three type 1, seven type 2 and one due to pancreatitis) versus 10 non-diabetic donors. They concluded that the cumulative replicative life span: (1) decreases as a function of donor age for controls but only from some diabetics, (2) is not different from random subsites of the same biopsy, (3) is not affected by freeze-thawing of either tissues or cells, or by the calendar time during which the cells were tested, (4) is affected by different lots of foetal bovine sera.

From the studies discussed it is concluded that fibroblasts cultured from human skin biopsies of diabetic subjects display inherent cellular defects. Three possibilities are presented and discussed by Vracko (16). He rejects the suggestions of an acceleration of cell senescence by an inappropriately set clock mechanism and that diabetics' cells are exposed to increased intensity and/or frequency of injury, and favours the possibility that fibroblasts of patients with diabetes mellitus are excessively vulnerable to injury and death. Although the mechanism(s) is unknown it is sufficient for the present study to conclude that human skin fibroblasts from diabetic subjects maintain inherent characteristics in culture.

C. Diabetes mellitus

There is still a controversy on the importance of environmental versus hereditary factors in the pathogenesis of diabetes mellitus and its complications. The diabetic state is in the majority of cases complicated by lesions in the eyes, kidneys, heart, nerves, and arterial vessels. Diabetes mellitus is divided into two major groups, the type 1 (insulin-dependent diabetes mellitus, juvenile onset diabetes (JOD)), and type 2 (non-insulin-dependent diabetes mellitus, maturity onset diabetes (MOD)). There is today

no clear distinction between the two types. It is generally agreed that the two major groups of idiopathic diabetes, type 1 and type 2, are genetically different. The hereditary mechanisms are not known. One subtype of type 2, maturity-onset diabetes in young people (MODY) (Mason syndrome), is characterized by autosomal dominant inheritance (19). For all types of diabetes it is proper to consider that diabetes mellitus per se is not inherited, rather it is the susceptibility to develop the disease which is transmitted (20). Even if the mode of genetic transmission is unknown for type 2 diabetes there is a very strong familial aggregation of the disease, as illuminated by twin studies (21).

D. Complications of diabetes mellitus: selected aspects.

Macroangiopathy

In diabetics macrovascular disease accounts for about 75 per cent of all deaths, mainly expressing itself as cardiovascular disease. The risk factors of arterial disease are essentially similar to those in the non-diabetic (22). The diabetic patients are, however, at a particular disadvantage because of at least three additional factors unique to their diabetic state: (a) presence of microangiopathy, (b) hyperglycaemia and its consequences, direct or indirect, and (c) hormonal aberrations underlying the altered metabolic state. Abnormalities in the haemostatic system (coagulation, fibrinolysis and platelet function) may also be relevant for the pathogenesis of vascular disease in diabetes (23, 24).

Microangiopathy

The capillary basement membrane (CBM) thickening is evident throughout the body, including muscle and skin capillaries, and clinically reflected principally in the retina and the kidney. A controversy has been devoted to whether the basement membrane thickness is normal at the time of clinical onset of juvenile

diabetes (25) or not (26). Present status favours the concept that basement membrane thickness is positively related to the level of glycemia (27). The CBM thickening might be influenced by the less soluble collagen in diabetic tissues (28, 29) caused by increased crosslink formation and to nonenzymatic glycosylation of collagen and other proteins (30). In a proposed model to explain the thickening of the basement membrane seen in diabetes, it is suggested (31, 32) that in diabetes there is a lack of the heparan sulfate proteoglycan and a compensatory synthesis of other basement membrane components as type IV collagen and laminin. The increased permeability is mainly attributed to the reduction in basement membrane heparan sulfate proteoglycan (33). The turnover of glycosaminoglycans is supposed to be normal, although an undersulfation of glycosaminoglycan chains is demonstrated (34). The regulation of synthesis of collagen, protein and glycosaminoglycans is therefore of importance for the understanding of microangiopathic complications in diabetes.

Hyperinsulinaemia

An association between hyperinsulinaemia and atherosclerotic vascular disease, in diabetics and non-diabetics, has been proposed (35). Three studies have demonstrated plasma insulin levels as an independent risk factor for atherosclerotic vascular disease in non-diabetic populations (36, 37, 38). A significantly higher insulin-glucose ratio is reported (39) in diabetics who had clinical and electrocardiographic evidence of coronary atherosclerosis, although no significant difference was noted between fasting levels of insulin in diabetic patients with and without atherosclerosis. A recently published prospective study (40) provides the first link between hyperinsulinaemia in diabetics and later cardiac lesions. However, in 120 consecutive patients, including 10 known diabetics, undergoing diagnostic coronary arteriography, neither fasting plasma insulin levels nor insulin-glucagon ratios

were correlated to the presence or extent of coronary atherosclerosis (41). In accordance with the prevailing hypothesis on the pathogenesis of atherosclerosis (42, 43) experimental studies have been performed on cultured smooth muscle cells. Insulin in concentrations of 10 to 10,000 μ units/ml was found to stimulate proliferation of smooth muscle cells from thoracic aortas of monkeys, and a significant linear relationship was noted between the logarithm of the insulin dose and cell growth. However, the highest concentration of insulin produced only 50 per cent of the effect of 5 per cent monkey serum (44). A similar effect of insulin (10-10,000 mU/l) on the proliferation of cultured human arterial smooth muscle cells has been reported (45). In this study the highest concentration of insulin had only a third of the effect of 10 per cent foetal calf serum on cell growth. Serum from diabetic rabbits (46) and human diabetics (47) increases the outgrowth of smooth muscle cells from cultures of rabbit thoracic aortas. The growth stimulating effect of human diabetic serum was attributed to growth hormone and not to insulin (48, 49). Human diabetic serum has also stimulated the cell growth of non-diabetic human smooth muscle cells as well as diabetic and non-diabetic human skin fibroblasts (50). The different results of insulin effects from these studies might be due to species heterogeneity. For the *in vivo* situation a hormonal-growth factor interrelation is probable, and the hormonal effects of growth hormone and insulin may both be mediated by somatomedins, as in foetal growth (51). Effects of hyperinsulinaemia on low density lipoprotein should also be considered (52-55, 22).

Hyperglycaemia

The connection between hyperglycaemia and atherosclerosis has been discussed for a long time, but there are still only indirect clues for an interaction of glucose with the blood vessel wall. The population study in Tecumseh indicated that even modest hyper-

glycaemia, without clinical diabetes, was associated with increased prevalence of arterial disease, cerebral or peripheral (56). In the Whitehall study, those participants with the highest 2 per cent of the blood glucose levels had a doubling of coronary heart disease mortality after five years. Renewed analysis after another two and a half years showed a sharp increase in coronary heart disease mortality with increasingly high levels of glycaemia, mostly above the 95th percentile (57). In the Framingham study, the conclusion was drawn that a unique factor must be involved to explain the increased morbidity in diabetic coronary heart disease (58). As a glucose effect on coronary heart disease is revealed only in a few of the vast body of population studies available, one might be inclined to accept a specific diabetic macroangiopathy in combination with atherosclerosis as proposed by Ledet (59, 60). In peripheral arteries a more direct association between glucose and atherosclerosis has been demonstrated in patients with intermittent claudication. Angiography demonstrated a highly significant association between the severity of atherosclerosis and blood glucose measured after an oral glucose load (61). The mechanisms for any glucose effect contributing to the development of macroangiopathy are essentially unknown.

Platelets

The impact of fibrinolysis, coagulation and platelet function in the pathogenesis of diabetic macro- (and micro-) vascular complications has been considered in several studies (62-65). In the "response to injury" hypothesis of atherosclerosis the key event is proliferation of the smooth muscle cell in the arterial wall (42). A growth stimulating factor released from platelets was therefore proposed to participate in the development of atherosclerosis (43). This was supported by other studies (66, 67). The growth stimulating factor, platelet derived growth factor (PDGF), is released from platelet alpha-granules, and responsiveness of

dermal fibroblasts was reported (68). Subsequent studies have characterized the molecule and its receptor, indicated several effects, and made known that PDGF is the predominant, but not the only, mitogenic substance present in human platelets.

Limited joint mobility

The observation by Lundbaek (69) on five adults with long-standing insulin-treated diabetes whose hands had become painful and stiff has evoked renewed interest since it was reported that periarticular connective tissue thickening indicated a high risk for microvascular disease in young diabetics (70). Life-table analysis indicated an 83 per cent risk for microvascular complications after 16 years of diabetes if joint limitation was present, as compared to 25 per cent risk if absent. The cheiroarthropathy may also occur in type 2 diabetics, and was also suggested to be associated with fibrous breast lumps in type 1 diabetic women (71). There is evidence for metabolic mediation of these connective-tissue changes, since improved control of diabetes changes the dermal manifestations for the better (72). Limited joint mobility is not related to non-enzymatic glycosylation of collagen (73).

E. Connective tissue components

Collagen

Synthesis and types: Collagen is the major molecule of most connective tissues and constitutes nearly one-half of the total body protein in humans. Cultured human skin fibroblasts (HSF) were reported to produce collagen and non-collagen protein in 1964 (74). There are five structurally and genetically distinct collagens with variations in tissue distribution (75). Type I: bone, tendon, skin, dentine, ligament, fascia, arteries and uterus. Type II: hyaline cartilage. Type III: skin, arteries and uterus. Type IV: basement membranes. Type V: basement membranes and perhaps

other tissues. Since the ability of a cultured human skin fibroblast cell to produce both type I and type III collagen simultaneously was demonstrated (76) these cells have been used for studying regulation of collagen biosynthesis (77) in normal and diseased cell lines.

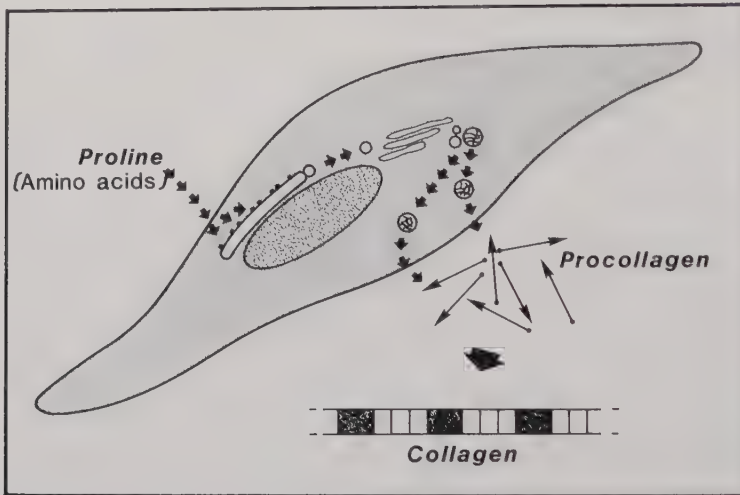


Fig 1. Elaboration of collagen. Amino acids entering the fibroblast are synthesized into polypeptide subunits at the ribosomes. The polypeptide chain is processed by modifying enzymes to procollagen. In the Golgi apparatus, the molecules appear to be packaged in vesicles for transport to the extracellular spaces. Extracellular changes, molecular assembly and cross-linking complete the formation of mature collagen fibers.

(Drawings, figs 1-3, by Lars Jansson.)

Glycosaminoglycans (GAG)

The connective tissue polysaccharides (glycans), deposited in the extracellular space, are called glycosaminoglycans (GAG) (formerly mucopolysaccharides). The name refers to their content of hexosamine residues, glucosamine or galactosamine. All of them, except hyaluronic acid, are sulfated. The glycosaminoglycans are

bound to protein in connective tissue. The large polysaccharide-protein complexes, proteoglycans, are held in position in the extracellular space by entanglement with a network structure of protein fibres, essentially collagen. These macromolecules give the tissue its mechanical properties and are essential for the transport of macromolecules through connective tissue. There are multiple interactions of the extracellular matrix components proteoglycans, collagen and fibronectin (78).

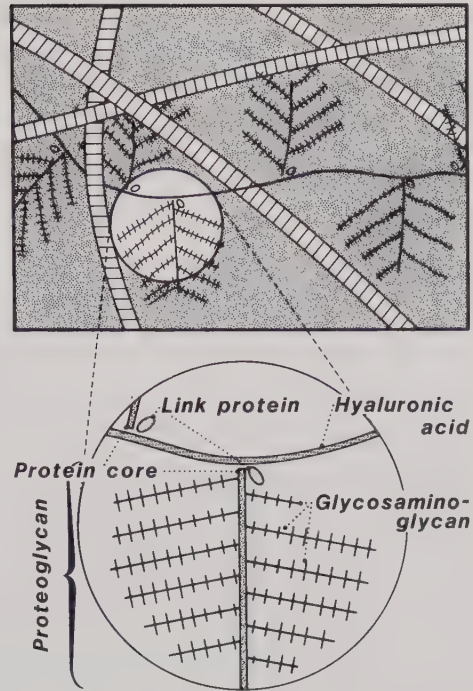


Fig 2. Proteoglycan monomer and aggregate in a network of collagen.

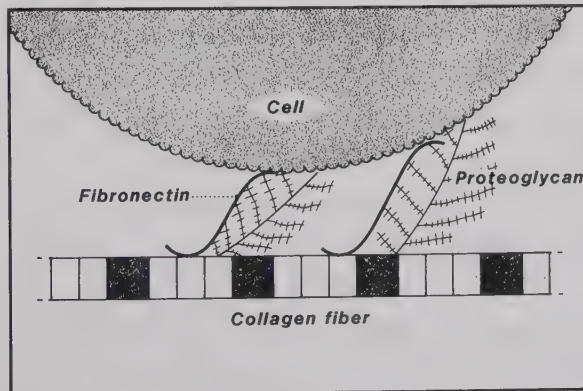


Fig 3. Collagen fibers contain specific sites to which the fibronectin molecule binds. The fibronectin molecule contains a region that recognizes collagen and another region that recognizes the cell surface. Proteoglycans are also present and stabilize the interaction.

It has been suggested that glycosaminoglycans participate in diabetic kidney disease, initially demonstrated as an increase of all GAGs but in particular heparan sulfate, in kidney cortex and medulla (79). Also, the lowered content of GAGs in the skin of diabetic rats was almost entirely restored by insulin administration (80, 81). An alteration in subunit composition was supposed to be the basis for the defective filtration function in diabetes (82). More recently, it has been proposed that the basement membrane becomes more porous due to increased degradation or inadequate synthesis of heparan sulfate proteoglycan (31).

Heparan sulfate is the only sulfated GAG detectable in the glomerular basement membrane in rats (83). Heparan sulfate and chondroitin sulfate are also found to be the major GAGs of aortic myomedial basement membrane-like material isolated from rabbit aortic myomedial cells in culture (84). The specific role of GAGs in arterial extracellular matrix is unknown, although the demonstration of a marked propensity of dermatan sulfate to bind low-density lipoproteins is interesting (85).

F. Lysosomal enzymes

Lysosomes are membrane-bound organelles, containing several acid hydrolases as β -hexosaminidase and β -glucuronidase. The medium surrounding cultivated skin fibroblasts contains lysosomal enzymes secreted by the cells. Cultivated skin fibroblasts have become a major tool for the biochemical investigation of lysosomal storage diseases, since fibroblasts in cell culture express the basic defects of these disorders (86, 87). Wolinsky and Fowler have proposed a role for lysosomes in atherosclerosis (88). They suggested an intralysosomal accumulation of intra- and extracellular macromolecules through different mechanisms. In streptozotocin and alloxan induced diabetes mellitus in rats the specific activities of all aortic hydrolases were found to be significantly decreased. Complete restoration of normal enzyme levels was a-

chieved by insulin treatment (89). Lysosomal enzymes, when present in low concentration, could also act as mitogens and thus participate in the regulation of cell proliferation within the vascular wall (90).

In diabetes mellitus increased serum activity of the lysosomal enzymes, β -glucuronidase and β -hexosaminidase (= N-acetyl- β -glucosaminidase), has been found. A high correlation between blood sugar level and serum β -hexosaminidase activity was found in diabetics (91), and the enzyme level seemed to correlate with micro- and macroangiopathy (92). An increased serum level of β -hexosaminidase has in another study been found only in type 2 diabetics, treated with oral hypoglycemic agents or diet, and no correlation was found with retinopathy (93). The role of lysosomal enzymes, if any, in diabetic macro- and/or microangiopathy remains unclear.

3. PRESENT INVESTIGATION

A. Aims

The aim of the present study was to create a feasible model system to provide in vitro information relevant to the pathophysiology of diabetic complications. The idea was to develop a cell culture system using human skin fibroblasts from non-diabetic and diabetic donors. Connective tissue components were to be measured as markers of the synthetic ability of fibroblasts. The aim of the present study was to provide information on the following questions:

1. Does platelet derived growth factor(s) have any effect on macromolecule production by cultured human fibroblasts, and if so, are there any species differences?
2. Is collagen and total protein production by cultured cells affected by glycosaminoglycans?
3. Do insulin and/or glucose have any effect on the macromolecule production by cultured fibroblasts?

4. Can insulin and/or glucose levels affect lysosomal enzymes in cultured cells?

5. Is it possible to demonstrate any differences in protein production, glycosaminoglycans or lysosomal enzyme activity between cells from non-diabetic and diabetic donors?

B. Material and methods

Cell cultures (I-V)

Optimal conditions for the cell culture system were developed using human embryonic skin biopsies.

All skin biopsies in the present investigation (I-V) were transferred into cell culture medium immediately after removal. Skin punch biopsies, 2-4 mm in diameter, were taken without anesthesia by drilling with a cylindrical knife. The biopsies were 2-4 mm in depth, and the biopsy site was standardized to the skin of the medial aspect of the left upper arm. This biopsy site is assumed to be the most protected and uninjured skin area of the human body. The wound was covered with adhesive plaster and no complications are reported. The skin fragment was transferred to a dry sterile plastic petri dish and cut into small (0.5 x 1 mm) pieces using an ordinary sterile surgical blade. A finding, however, not used for this study, was that skin fragments were able to survive for 48 hours in culture medium before further processing. The fragments, usually 10-20, were placed on the dry bottom of a 25 cm² plastic culture flask using a hooked glass Pasteur pipette. The vessel was turned upside down, 5 ml culture medium was added and the vessel was placed in 37°C. The biopsies were thus left uncovered with medium for 1-1.5 h to ensure adhesion before the vessel was turned again, to be left untouched during 10 days for outgrowth. During outgrowth the medium was changed twice a week.

Outgrowth of cells was generally noted after 2-3 weeks around several pieces of each biopsy. A confluent cell monolayer in the

primary flask was present after another 2-3 weeks. In the following subculturing a 1:2 split ratio was used; thus one passage = one population doubling. A gentle trypsinization with 0.05 per cent trypsin-0.02 per cent EDTA or 0.025 per cent trypsin-EDTA was used for subculturing. The cell cultures were grown in 37°C in air atmosphere in synthetic liquid medium; minimum essential medium with Hanks' salts and 20-25 mM HEPES buffer, supplemented with L-glutamine 585 mg/l, non-essential amino acid mixture 1 per cent, and gentamicin 100 mg/l. In addition 10 per cent foetal bovine serum, heat-inactivated, was used. Enough serum for one year's use was purchased at one and the same time to minimize using different batches. In addition, at every change of serum batch we tested several samples for growth stimulating capacity to ensure minimal variations.

Cell numbers, growth and viability (I-IV)

Determinations of deoxyribonucleic acid (DNA) were performed in each experimental dish to estimate the cell number (I-IV). There were two reasons for this: 1) DNA could be quantitated from the same cell population as the other parameters used; and 2) in our experience, the variation of DNA measurement was less than that for cell number. Values for DNA per cell are similar for subcultivations and at different times within each subcultivation. Thus, cell number and DNA are considered interchangeably throughout the study. The DNA method used was described by Fujimoto, Teague and Williams (94). In short: medium is removed, the cell layer rinsed and air-dried (and usually stored at -20°C for later analysis). The cell layer is extracted with trichloroacetic acid (TCA) and scraped from the dish with a rubber or teflon policeman. The method is based upon the formation of a fluorescent product due to the specific reaction of 3,5-diaminobenzoic acid with deoxyribose, released from hydrolysis of DNA. The fluorescence was

measured in a Perkin Elmer fluorescence spectrophotometer (MPF-43A) with a xenon power supply (Perkin Elmer 150).

Cell growth was assayed by the incorporation of ^3H -labelled thymidine into cellular DNA as described by Witte et al. (95). ^3H -thymidine incorporation is supposed to parallel DNA quantities in growing cells.

Cell counting was performed in a Bürker haemocytometer chamber. Samples of cells in a 0.1 mm^3 volume were routinely counted. A multiplication by 10,000 thus gives the number of cells per ml.

Measurement of cell viability was made by the trypan blue dye exclusion test according to Pattersson (96).

Collagen and total protein determinations (I-IV)

We have used radioactive proline as a precursor for fibroblast procollagen synthesis. By this technique 94 per cent of collagen produced is found in the medium and 6 per cent in the cell pellet. Under optimal conditions, collagen comprised 24 per cent of all protein in the medium (97). The incorporation of ^3H -proline by the cells into extracellular collagen was measured according to the method described by Peterkofsky and Diegelmann (98). The method used is based on the ability of collagenase to cleave collagen into acid-soluble peptides. After treatment with trichloroacetic acid (TCA), protein was dissolved in sodium hydroxide and pH adjusted with N-2-hydroxyethyl-piperazine-N'-2-ethanesulphonic acid (HEPES)-hydrochloride, and N-ethylmaleimide (NEM) was added. 10 units of chromatographically purified collagenase from *Clostridium histolyticum* (Worthington CLSPA), were added to HEPES-HCl+NEM-Calcium solution including test samples. To each test sample a control tube was also included containing saline as a substitute for collagenase. Tritium radioactivity was counted in Biofluor (New England Nuclear) on a Packard B 2450 liquid scintillation spectrometer. Quench correction was made by external standard. The counts per minute (CPM) from collagenase containing vials were subtracted

by the CPM from appropriate controls, and the net result was considered to represent the portion of extracellular protein that was collagenase-digestible, i.e., collagen. Clostridial collagenase degrades the helical regions in native collagen preferentially at the Y-Gly bond in the sequence Pro-Y-Gly-Pro where Y is most frequently a neutral amino acid. The chromatographically purified collagenase contains essentially no clostripain or tryptic activities and less than 10 per cent caseinase activity. Besides, about 80 per cent of the non-specific proteolytic activity is inhibited by N-ethylmaleimide (98). The amino acid sequence, for which clostridial collagenase has a specificity, is found almost exclusively and with great frequency in collagen (99). The method could thus be used in the presence of other proteins. In most experiments foetal bovine serum was present in the samples, and in the serum-free experiments similar concentrations of serum were added before analysis.

The ^3H -proline activity incorporated into total extracellular protein was estimated as part of the above procedures by measuring the radioactivity incorporated into trichloroacetic acid precipitates.

To further confirm the results obtained we have performed similar experiments but have used microwell tissue culture plates (A/S Nunc) and measured collagen production according to Webster and Harvey (100). This assay is based on the purification of ^3H -labelled collagen by precipitation with salt at acid and neutral pH successively. The extraction of collagen from cell cultures with pepsin together with salt purification renders the assay specific to at least 90 per cent for native collagen molecules. Total protein cannot be assayed by this method. We found equivalent results by both methods for collagen determination.

In this study (I-IV) collagen and total protein were determined in the medium. As the extracellular product was measured, we have considered the term production most appropriate for the re-

sult of intracellular biosynthesis and secretion into the medium. There is no uniformity for these terms in the literature.

Glycosaminoglycan (GAG) determinations

Determinations of sulfated glycosaminoglycans (S-GAG) were performed according to Wasteson et al. (101). This method is based on precipitation with cetylpyridinium chloride of polysaccharide samples applied to squares on strips of filter papers, and the $^{35}\text{SO}_4$ labelling into S-GAG is counted.

An alternative method was used for the simultaneous determination of radiolabelled hyaluronic acid (HA) and S-GAG (102). In these experiments ^3H -glucosamine was used as precursor. The extracellular medium was proteolytically digested using papain and precipitated with cetylpyridinium chloride (CPC) into cellulose filtration membranes. The hyaluronic acid was eluted from the membrane with hydrochloric acid (HCl). Further elution with HCl rendered the eluate free from radioactivity. The membrane with the CPC precipitate containing sulfated glycosaminoglycans was dissolved in Biofluor (10 ml) and counted in a Packard scintillation spectrometer.

Lysosomal enzyme determinations (V)

The lysosomal enzyme β -hexosaminidase was analyzed using p-nitrophenyl- β -N-acetylglucosaminide as substrate (103).

C. Results and discussion

Growth factor(s) from platelets (I)

When we planned our study on the effects of platelet-derived growth factor(s) in cell cultures it was known that the platelet-derived growth factor (PDGF) was released from the platelet α -granules, and that it could stimulate in cultured arterial smooth muscle cells cell proliferation, uptake and storage of lipids, and the synthesis of connective tissue materials (104).

For experiments in paper I growth factors were derived from human platelets (105) after a 120 min spontaneous efflux at 37°C. The platelet supernatant thus obtained contained materials from the alpha-granules, including PDGF and other putative growth promoting factors. In our system the efflux of growth stimulating activity was similar to the efflux of β -thromboglobulin and ^{14}C -norepinephrine (106).

Human skin fibroblasts (HSF) were obtained and cultured as described in Methods. Rabbit aortic fibroblasts were obtained from adventitial fragments of thoracic aortas and cultured as HSF. Rabbit aortic smooth muscle cells (SMC) were prepared and cultured as described by Ledet (47). Pilot experiments were performed to test the vitality of cells for prolonged periods in serum-free medium, and we found only minor (10 per cent) signs of decreased viability during a four-day period. A two-day period in serum-free medium was sufficient to obtain a growth-arrested state in all cell types. The platelet-derived supernatant was added at various concentrations for another 2 days in serum-free medium. The findings of a dose-dependent relation between added amount of supernatant and extracellular collagen production were similar in the three cell types. The cell number as assessed by DNA remained unchanged. ^3H -thymidine incorporation into DNA was, however, significantly increased with increasing concentration of platelet supernatant. Similar results were obtained in the various cell types. The discrepancy in findings was evaluated by flow cytometry. We found only 1 per cent of the cells (SMC) to be in the phase of DNA synthesis (S), 94 per cent of the cells being in pre-S period (G0/G1) and 5 per cent in post-S predivision period (G2). These findings were not changed by the highest amount of platelet supernatant used. Thus, it was concluded that a minor proportion of cells may have entered or traversed the S phase and might be responsible for the increase in ^3H -thymidine incorporation. In a simultaneously published report on the effect of PDGF

on cultured rat calvaria it was found that there was a 20-300 per cent increased incorporation of ^3H -thymidine into acid insoluble residues while the DNA content increased only from 9.2 μg to 14.5 μg (107). In that study it was also noted that PDGF caused a dose-dependent stimulation (0.6-60 ng/ml of PDGF) of ^3H -proline incorporation into non-collagen protein and a small stimulation of its incorporation into collagenase-digestible protein.

Owen et al. have demonstrated stimulation of amino acid transport and ^3H -leucine incorporation as an early effect of PDGF on quiescent human fibroblasts. Secondly, the action of PDGF in amino acid uptake and ^3H -leucine incorporation was found to be brought about by PDGF alone and did not require simultaneous or subsequent presence of plasma components or other hormones (108). This finding is in contrast with the effects of PDGF on DNA synthesis and cell proliferation, in which the presence of plasma factors or hormone mixtures is required. It is also suggested that a fundamental action of PDGF could be to enhance the sensitivity of cultured fibroblasts to epidermal growth factor (EGF) (109). EGF has been demonstrated to be a mitogen for many fibroblastic cell lines. The concentrations of EGF in these experiments have been much higher (4-20 ng/ml) than the concentration found in human serum. Therefore, EGF alone is not likely to be mitogenic, but must function synergistically with other factors in serum to promote proliferative growth.

Human whole blood serum, clotted overnight at 37°C to ensure complete release of alpha-granule components, has recently been found to contain 17.5 ± 3.1 ng/ml PDGF and the calculated amount of PDGF per platelet was 0.073 ± 0.016 ng/ 10^6 platelets (110). It is now also known that PDGF is the predominant but not the only mitogenic substance present in human platelets (111).

During the last 2-3 years knowledge of the biological properties of PDGF has grown considerably. Within minutes PDGF stimulates phosphorylation of tyrosine, and of several cytoplasmic

proteins, increases phosphatidylinositol turnover and arachidonic acid metabolism, causes the appearance of membrane ruffles and microvilli, reduces ^{125}I -epidermal growth factor binding, increases amino acid uptake, and stimulates Na-K pump activity. Over a period of hours PDGF increases the number of low density lipoprotein receptors and somatomedin receptors, induces chemotaxis by vascular SMC, fibroblasts, and monocytes, and increases the rates of fluid-phase pinocytosis and protein synthesis. Finally, over a period of days, PDGF stimulates cell proliferation and maintains cell viability. (For an extensive review, see Bowen-Pope (112)).

Our finding of platelet growth factor-mediated stimulation of collagen production is in accordance with the accumulated knowledge and supported by the demonstration of many other cell processes stimulated by PDGF, many of them soon after exposure of the cell to PDGF and before initiation of DNA synthesis.

Human platelet alpha-granule proteins also include platelet factor 4, β -thromboglobulin (BTG), and thrombospondin. The plasma BTG level is reported to vary with the metabolic regulation in diabetics (113). Therefore, studies are in progress at our laboratory to investigate whether the diabetic state as such, or the degree of metabolic control, might influence the platelet release of growth factors and also the collagen production by cultured cells.

Collagen and total protein production

Effects of insulin (III)

Insulin is naturally of particular interest in the pathophysiology of diabetes mellitus as well as in the vascular complications. It has been proposed that insulin contributes to the development of macroangiopathy in diabetics (35). The demonstration of specific binding sites for insulin in human lymphocytes and cultured human skin fibroblasts (114) stimulated in vitro studies of

clinical disorders. Pork, beef, and human insulin were found to be equipotent in binding to human skin fibroblasts.

Various effects of insulin have been studied in cultured human fibroblasts from diabetics and non-diabetics in order to elucidate the basic genetic defect(s) in diabetes mellitus (115-122). From these studies it seems reasonable to assume, despite the relative in vivo insulin resistance, that cultured fibroblasts from diabetic subjects have normal insulin sensitivity and an absence of intrinsic (genetic) cellular defect(s) in insulin binding or insulin action on glucose transport and metabolism. However, although cultured fibroblasts are insulin responsive, they are not major insulin target cells.

We studied the effect of insulin on proline incorporation into collagen and total protein (paper III). The insulin concentrations used were 10^2 to 10^5 mU/l. (One U corresponds to 0.04167 mg; 10^2 mU/l insulin = 4 ng/ml = 0.7 nmol/l.) The 10^2 mU/l insulin level is frequently occurring in normal man. The 10^3 mU/l level also occurs in some individuals in the postprandial state. Furthermore, it is not infrequent in type 1 diabetics since the mean 24 h concentrations of free insulin are reported to be 3.5 times higher in type 1 diabetics than in non-diabetics. The mean total insulin integrated concentration was more than 20 times in excess of total insulin concentrations in non-diabetics (123). The two highest concentrations used are pharmacological (10^5 mU/l = 4000 ng/ml = 667 nmol/l). Insulin is generally supposed to accelerate growth in several types of cells from various species. A common result has been that only pharmacological doses of insulin (usually 1 μ g/ml) exhibited mitogenic activity, and that these doses gave only small effects if compared with those obtained with other growth promoting agents such as epidermal or fibroblast growth factor (124). In our experiments there were no indications of increasing cell numbers (DNA per dish) corresponding to insulin additions. The insu-

lin used (Actrapid, Novo) was of the porcine monocomponent type, the purest insulin available.

In cells from non-diabetic subjects we found a significant increase of ^3H -proline incorporation into extracellular collagen at an insulin concentration of 10^3 mU/l. This is in close agreement with findings at similar doses on cultured rat calvaria. Canalis (125) noted a significant increase of ^3H -proline into collagen with insulin concentrations of 100 ng/ml (2500 mU/l) for 24 h. Using isolated chondrosarcoma chondrocytes from tumors maintained in Sprague-Dawley rats, Bembenek et al. (126) found a stimulatory effect of insulin on the incorporation of ^3H -proline into collagen. They too, used similar concentrations of insulin (range 0-180 nmol/l) and also found a decrease of the stimulatory effect with the highest insulin levels. The effects of insulin on protein synthesis were studied by Airhart et al. in serum-free cell cultures of skeletal muscle cells and cardiac muscle cells from chicken embryos (127). The cardiac muscle cells were found to have a dose-response relation in protein synthesis with a peak at 8.0 nmol/l of insulin and no further stimulation with insulin concentrations up to 800 nmol/l. Their results are in close similarity to our finding of a maximal insulin stimulation of cultured human fibroblasts at 7 nmol/l. Airhart et al. reported a similar finding in the skeletal muscle cells in response to insulin in the physiological range. Higher insulin concentrations (80-800 nmol/l) caused a substantial unexplained drop in the protein synthesis by these cells. This was also a notable finding in our study on collagen production by fibroblasts from diabetic subjects and was even more pronounced in the total protein production by these cells (Figs 4a-c), in contrast to the insulin effect on protein synthesis in cells from non-diabetic donors. This finding still lacks an explanation.

We performed additional experiments on insulin effects on collagen and protein production in the presence of 10 per cent

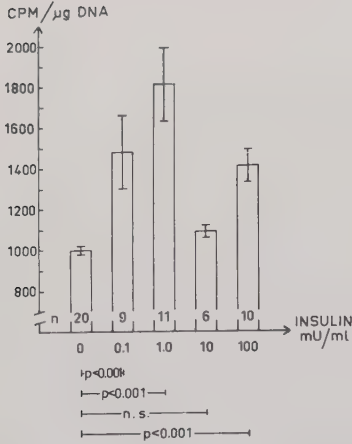


Fig 4a. Insulin influence on 3 H-proline incorporation into extracellular total protein. Fibroblasts from five type 1 diabetics. n = number of pooled dishes. M + SEM. Glucose concentration 5.6 mmol/l.

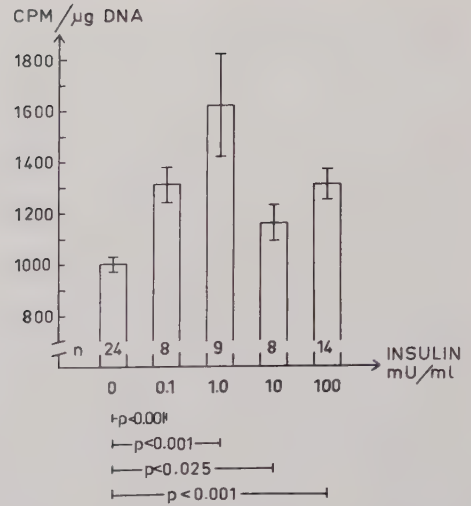


Fig 4b. Fibroblasts from type 2 diabetics. Otherwise as in fig 4a.

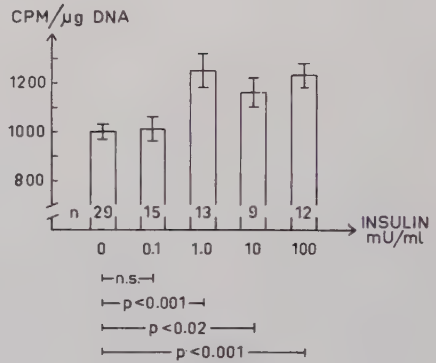


Fig 4c. Fibroblasts from non-diabetics. Otherwise as in fig 4a.

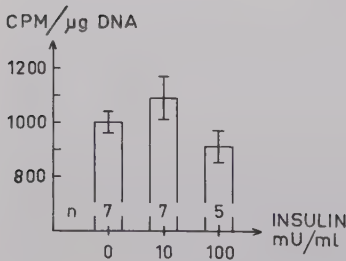


Fig 5a. Fibroblasts from two non-diabetic subjects. Insulin effect on collagen production at 5.6 mmol/l glucose and 10 per cent FBS.

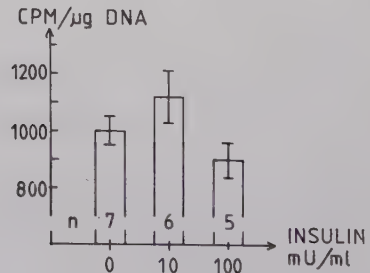


Fig 5b. Total protein production. Otherwise as in fig 5a.

foetal bovine serum. In the presence of serum, the effect of insulin was totally abolished (Figs 5a and b). This is consistent with the previously discussed findings of insulin effect on cellular growth. Also, high concentration of insulin (10^4 mU/l) did not stimulate growth in smooth muscle cells from rat aortas to the same degree as medium containing 10 per cent serum (128).

In our studies on cells from type 1 diabetic subjects insulin in serum-free medium caused a dose-response related increase in collagen production similar to the results with non-diabetic cells. However, the stimulatory effect of the lowest insulin concentration was clearly higher in cells from diabetics, as was the insulin effect in general. In cells from type 2 diabetics a similar higher sensitivity to insulin additions was noted. This is also valid for the total protein produced extracellularly as shown in figures 4a-c.

Similar experiments were performed with glucose-enriched medium, 38.9 mmol/l. The discrepancy between diabetic and non-diabetic cells at the lowest insulin stimulation was now absent. No significant differences were thus noted in collagen production for any cell type, between 0 and 10^2 mU/l of insulin. Otherwise, the collagen production was rather similar at various insulin levels for the three cell types, as illustrated for type 1 diabetics (Fig 6).

Fig 6. Collagen production by fibroblasts from one type 1 diabetic, studied at high glucose concentration (38.9 mmol/l). n = number of dishes. $M \pm SEM$.

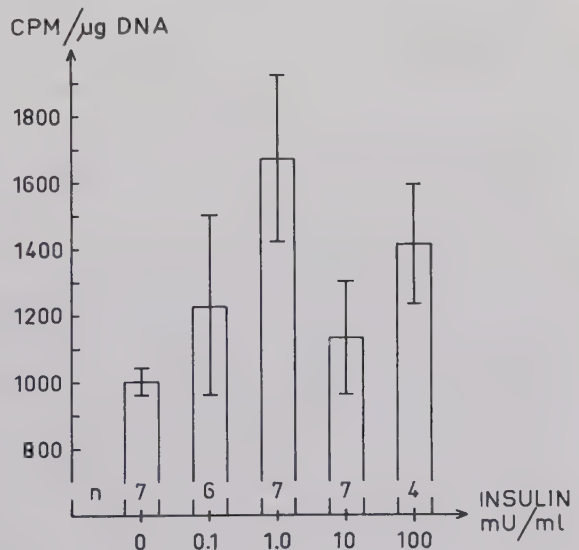
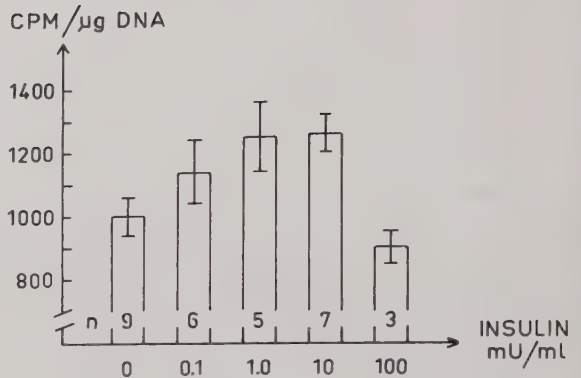


Fig 7. Total protein production by fibroblasts from two non-diabetic subjects. Influence of insulin at 38.9 mmol/l glucose concentration. $M \pm SEM$.



The total protein production at the high glucose level was not very different in the non-diabetic cells (Fig 7) as compared to the 5,6 mmol/l glucose level. In both diabetic cell types insulin had no effect on the total protein production at the high glucose level. This finding stands in contrast to the results obtained at physiological glucose level (Figs 4a and 4b). There is no similar report in the literature, and our observations suggest the possibility of a disturbed insulin effect on the total protein synthesis at high glucose level in cells from diabetics.

Although there is no explanation so far for the differences between cells from diabetics and non-diabetics the in insulin regulation of collagen production at physiological glucose level, it might be the reflection of an intracellular metabolic effect rather than an effect of insulin receptors and/or insulin binding to the different cell types(see above). It remains to be explored whether the stimulatory effect of insulin contributes to the production of type I and type III collagen in a similar way. Diabetic serum has been demonstrated to enhance only the production of procollagen type I in arterial myomedial cells (129).

It is recognized that the insulin effects discussed may be related to one or more of several processes, i.e. tracer uptake, secretion and degradation of collagen. However, in bone cultures

from fetal rats, insulin appeared to have a direct effect on collagen synthesis and not on collagen breakdown (130).

It is concluded that recovery of ^3H -proline labelled collagen and total protein in the medium was clearly stimulated by insulin at physiological levels. Fibroblasts from type 1 and type 2 diabetics displayed a higher sensitivity to low insulin levels than cells from non-diabetics. High glucose concentration in medium disturbs this insulin effect, mainly on the synthesis of total protein.

Effects of glucose (IV)

There are few studies on human cells in culture to elucidate effects of factors in the abnormal metabolic environment associated with the diabetic state.

Cell growth: It has been reported that high glucose media enhanced proliferation of human skin fibroblasts (HSF) in culture (131). No differences in growth behaviour were apparent between fibroblasts from non-diabetic and type 1 diabetic donors. High (optimal 18 mM) glucose media were found to enhance the proliferation of HSF only after 12 days of exposure. The experimental media were changed daily to maintain a constant glucose concentration. In our laboratory we have observed significant stimulation of HSF proliferation induced by frequent renewal of media (132). This factor may contribute to their results. In any case, no increased proliferation due to glucose was observed within 6 days as was confirmed by us. We cultured HSF and studied the ^3H -thymidine incorporation into DNA 48 h after renewal of media containing various amounts of glucose (Fig 8). Another study, using fibroblasts from human infant abdominal skin, reported a significant increase in cell proliferation after 3 days at glucose 5 mg/ml (27.5 mM) as compared to 1 mg/ml medium, but cell morphology was not found to be different until after 3 days in high glucose media (133). Thus, it is concluded that no major alterations in cell

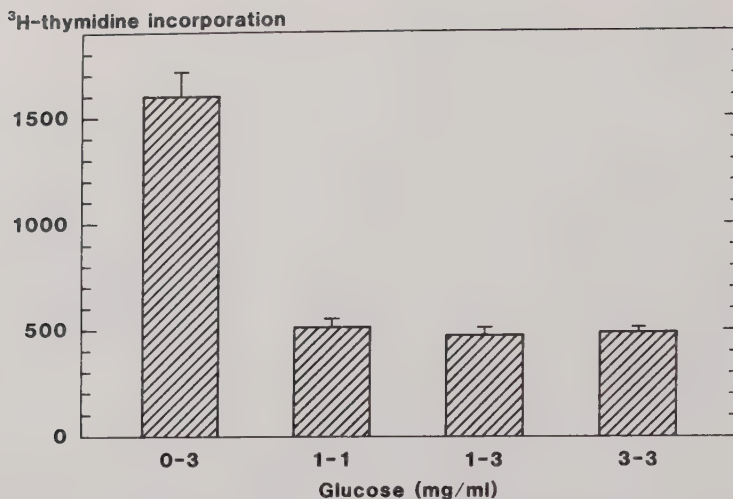


Fig 8. Growth effects of glucose on non-diabetic human skin fibroblasts. Preincubation at 0, 1, and 3 mg/ml for 24 h was followed by 24 h exposure to higher or similar glucose levels, when ³H-thymidine incorporation was measured. One cell line was used. Eight dishes in each test. $M \pm \text{SEM}$ is denoted.

proliferation or morphology are noted during a 48 h incubation at various glucose concentrations, as used in this study. Another major concern in experimental design (131) is the variation of the glucose concentrations during incubation. The results of media glucose determinations at various incubation periods are shown in table 1. The predetermined glucose concentration is given at time 0. No important changes in glucose levels were found.

Collagen: We investigated the effects of glucose on collagen and total protein production (IV) at glucose concentrations 1, 3, 5, and 7 mg/ml (5.6, 16.7, 27.8, and 38.9 mM). The production of collagen and total protein by fibroblasts from non-diabetics, type 1 and type 2 diabetics was measured. At the glucose concentration 5 mg/ml there was an increase of collagen production in both non-diabetic and type 1 diabetic cells. In contrast, cells from type 2 diabetics were not affected at all by glucose variations in the

Table 1. Glucose concentrations (mM) in the medium after 0, 10, 24, 48, and 72 h incubation with fibroblasts from non-diabetic and type 1 diabetic donor. M + SD of triplicates.

	0	10	24	48	72 h
Non-diabetic		5.9 $\pm$ 0.2	5.3 $\pm$ 0.1	5.4 $\pm$ 0.3	5.7 $\pm$ 0.2
Type 1	5.8 $\pm$ 0.1	5.5 $\pm$ 0.2	5.3 $\pm$ 0.1	5.5 $\pm$ 0.2	5.4 $\pm$ 0.1
Non-diabetic		15.4 $\pm$ 0.3	14.8 $\pm$ 0.2	15.3 $\pm$ 0.8	15.1 $\pm$ 0.9
Type 1	14.8 $\pm$ 0.2	14.8 $\pm$ 0.8	14.2 $\pm$ 0.3	14.9 $\pm$ 0.6	15.0 $\pm$ 0.9
Non-diabetic		25.1 $\pm$ 0.6	24.2 $\pm$ 0.2	25.0 $\pm$ 0.6	25.8 $\pm$ 2.1
Type 1	23.8 $\pm$ 0.3	26.7 $\pm$ 2.9	22.8 $\pm$ 1.0	24.9 $\pm$ 1.2	24.7 $\pm$ 2.0
Non-diabetic		35.8 $\pm$ 2.0	36.8 $\pm$ 0.3	37.2 $\pm$ 0.6	40.0 $\pm$ 2.2
Type 1	35.8 $\pm$ 0.4	38.2 $\pm$ 4.0	36.2 $\pm$ 1.0	38.0 $\pm$ 1.3	40.2 $\pm$ 0.6

medium, and the collagen production at glucose 5 mg/ml was different from type 1 diabetic cells. These differences remained whether calculations were based on grouped data from individual subjects or from pooled dishes. At lower glucose concentration (3 mg/ml) type 1 diabetic cells had a higher increase of collagen production, as compared to basal level, than both non-diabetic and type 2 diabetic cells. These differences were, however, found only in calculations based on pooled dishes. The increase in collagen production at 5 mg/ml glucose was not correlated to donor age or cell numbers (DNA) in the dish.

Total protein: Total protein production was not different between the three types of cells at any glucose level in calculations based on individual subjects. If calculations were performed on pooled dishes from the three groups there was an increase in all groups at a glucose concentration of 5 mg/ml, although no differences between the groups were found at any glucose level. It may thus be concluded that cells from non-diabetics, type 1 and type 2 diabetics respond in a similar manner to glucose in their

protein production, with a slight increase in production at 5 mg/ml (27.8 mM).

Discussion: In the only comparable study, by Villee and Powers, neither the incorporation of tritiated thymidine nor DNA content were affected by raising the glucose concentration of the medium (134). In the range of glucose concentrations 1-5 mg/ml they also found increasing amounts of collagen in the media of human non-diabetic skin fibroblasts. These results are in good agreement with our findings in similar cells.

Quite recently it was reported that high glucose concentrations in tissue culture stimulated total protein and collagen synthesis by bovine retinal vessel pericytes (mural cells), but reduced their growth curves and mitotic rates (135).

Our experiments do not permit conclusions about the effect of glucose. The findings on collagen and total protein production might be due to enhanced synthesis, decreased degradation or some other mechanisms. The observed difference between the cell types in our study makes it tempting to suggest a biologically relevant relation of collagen production to ambient glucose levels. Glucose can also affect the extracellular self-assembly of collagen into fibrils and the cross-linking (136, 137), which has further implications in the diabetic state.

Interactions: GAG-collagen (II)

Since cultured human fibroblasts also produce GAGs we were interested in the possible influence of extracellular polysaccharides on collagen production and the possible differences between fibroblasts from non-diabetics and diabetics. Heparin is increased in the aorta of alloxan diabetic rats (138). More recently, the levels of heparin and hyaluronic acid have been reported to be higher in the serum of human diabetics, while those of heparan sulfate, chondroitin-4-sulfate, and chondroitin-6-sulfate were lower and dermatan sulfate unchanged (139).

Heparin:In the experiments we compared effects of heparin, a sulfated glycosaminoglycan and dextran sulfate, an artificial homologue.

The addition of heparin to the culture medium (0.01-1.0 mg/ml) increased the collagen production. For comparison between fibroblasts from non-diabetics, type 1 diabetics, and type 2 diabetics we used only one concentration of heparin or dextran sulfate (0.1 mg/ml). Heparin caused a 50 per cent increase in ^3H -proline incorporation into extracellular collagen by the three cell groups. Addition of dextran sulfate also increased the collagen production in cells from non-diabetics and type 1 diabetics. The collagen production in cells from type 2 diabetics was unaffected by dextran sulfate addition, significantly different from the influence of heparin, and also different from the results in non-diabetic fibroblasts. The effects on total protein production were similar to the results on collagen production. In a report on human embryonic fibroblasts it was shown that dextran sulfate increased collagen markedly in both cell layer and medium, and protein in the cell layer, while that in the medium barely altered (140). Our results of heparin effect on collagen production are consistent with recent findings on smooth muscle cells (141). None of these reports offered any explanation for the findings.

Chondroitin sulfate: Chondroitin sulfate was reported to enhance only intracellular collagen synthesis in cultured pig arterial endothelial cells (142). In our study we noted a stimulatory effect of chondroitin sulfate on extracellular recovered collagen from human skin fibroblasts, thus demonstrating a difference between species and/or cell types.

Discussion: Further studies on the mechanisms of binding, uptake and secretion of proteoglycans and collagen must be done to interpret our results, especially with regard to fibronectin. Fibronectin binds to collagen, heparin and dextran sulfate. The interactions with collagen and heparin are independent of each

other (143). Alterations of the quantity of any of these components could have important effects on cell surface interactions. Anionic polymers such as dextran sulfate can bind strongly to the cell surface.

The response of type 2 diabetic cells in reaction to dextran sulfate remains unclear. There is, to our knowledge, no report on fibroblasts from type 2 diabetics in relation to cell attachment, spreading or fibronectin interrelationship. Cells from non-diabetics and type 1 diabetics were, however, reported to have marginal differences in these respects (144).

Glycosaminoglycans

Effects of insulin

In our laboratory GAG production was investigated in human fibroblasts. Cells were cultured from human embryonic skin, type 1 diabetics and non-diabetics. Insulin in various concentrations, 0 to 100 mU/ml, had no effect on the extracellularly produced sulfated GAGs (S-GAG) (145). The study was extended to fibroblasts from six type 2 diabetic subjects and gave similar results. These experiments were performed in serum-free medium at a glucose concentration of 5.6 mM. The insulin effect was also studied, in all three cell types, at high glucose concentration, 38.9 mM. No effect of insulin on sulfated GAG synthesis was noted. Absence of insulin effect on total GAG was also found with isolated diabetic and normal rat renal glomeruli (146), and human aortic smooth muscle cells (147). We found no differences in S-GAG or hyaluronic acid synthesis by fibroblasts from ten non-diabetic subjects, six type 1 diabetics and eight type 2 diabetics (unpublished). Similar findings on S-GAG were reported by Silbert and Kleinman (148), although they also noted an increased percentage of heparan sulfate in both groups of diabetic cell lines (type 1 and type 2). Our study on insulin effects included the synthesis of hyaluronic acid (HA) in fibroblasts from seven each of non-diabetic, type 1

diabetic and type 2 diabetic subjects. As with S-GAG, insulin had no significant effect. Insulin did not stimulate hyaluronate synthesis by rat chondrosarcoma fibroblasts (149) or human aortic smooth muscle cells (150).

It has been claimed that the glycosaminoglycan ratio S-GAG/HA is increased in early atherosclerotic lesions and may be changed by various human sera and by high cortisol concentrations (150). In our culture system this ratio was investigated in fibroblasts from six to eight donors each, non-diabetics, type 1 and type 2 diabetics. No differences were found in S-GAG/HA ratio at various insulin levels or at different glucose levels in any group or between the groups.

Thus, from our studies there is no evidence of an effect by insulin or glucose in glycosaminoglycan synthesis.

Lysosomal enzymes (V)

Lysosomal enzymes can be released into the medium in cultured human smooth muscle cells, endothelial cells and skin fibroblasts (151).

We investigated the intra- and extracellular activity of β -hexosaminidase in cultured fibroblasts from five type 2 diabetics and five non-diabetics. The groups did not differ, and there was no influence of insulin addition (concentrations 0.1 to 100 mU/ml). An almost 100 per cent increase of enzyme activity was found upon glucose elevation from 0 to 39 mM. Similar results were obtained in both non-diabetic and diabetic cells. The intracellular activity was unaffected by glucose concentrations. The observed increased levels of lysosomal hydrolases in human diabetic serum and urine, and the relation to acute changes in blood glucose (152), might thus be due to an increased release from cells exposed to elevated glucose concentrations in vivo. Our experiments were performed during a 24 h incubation. Experiments are in progress concerning long term effects of glucose and cell density.

The results display an effect of glucose, as above, but no influence of cell density on the ratio intra/extracellular lysosomal activity. In long term (six days) incubation of rat fibroblasts in insulin-enriched medium a significantly reduced release of β -hexosaminidase was reported (153).

D. GENERAL DISCUSSION

In this study a cell culture system was established, using human skin fibroblasts from non-diabetic, type 1 diabetic and type 2 diabetic subjects. This system has been used to investigate some metabolic processes of possible relevance to diabetes mellitus and its complications. Several reports have suggested possible inherent defects in cultured cells from diabetics; limitation in life span, plating efficiency and premature aging on a cellular level. From a clinical point of view, epidemiological studies have failed to disclose the pathogenesis of the various diabetic complications, although several risk factors have been identified. There is still uncertainty about the importance of the degree of glucose control in diabetes, as well as the genetic versus environmental influence on the development of complications.

A human cell culture system appeared to be appropriate for studies of numerous interacting factors under defined experimental conditions. Skin fibroblasts were chosen because these cells were obtainable from patients with diabetes mellitus. The studies were centered on effects of insulin and glucose.

Insulin. Insulin, in physiological levels, was found to increase the incorporation of radioactive labelled proline into collagenase-digestible protein (in the following referred to as collagen) in the cell cultures from diabetics and non-diabetics, without affecting cell proliferation. There were indications that the diabetic cells were more sensitive to this insulin action.

Interestingly, several diabetics are hyperinsulinemic, endogenously or due to treatment, and hyperinsulinemia has been pro-

posed as a risk factor for atherosclerosis, also in non-diabetics. Our results would favour this risk factor concept by demonstrating an insulin mediated increase of connective tissue matrix, due to increased production and/or decreased degradation, although we found no increase of the glycosaminoglycans. We found no differences between the different cell types on the S-GAG/HA ratio, which has been proposed to be increased in early atherosclerotic lesions.

Glucose. Our findings of glucose enhanced collagen production by cells from type 1 diabetics, and to a minor degree cells from non-diabetics, and no effect on fibroblasts from type 2 diabetics, indicate a difference between the two types of diabetic cells. The underlying mechanism is unclear. Differences in glycosylation of collagen and/or inherent cellular defects are some of several valid possibilities. The increase of collagen production in non-diabetic cells is also of interest.

Platelet derived growth factor. The effect of platelet-derived growth factor(s) on collagen production study merits further consideration with regard to the diabetic state. PDGF is known as a cell replication stimulator in vitro, but direct evidence of its supposed effects in the pathophysiology of atherosclerosis is still lacking. Our study indicates that PDGF(s) may also influence collagen production in connective tissue, as recently confirmed (154). This finding is of more specific interest to the diabetic state since the release of another platelet alpha-granule component, β -thromboglobulin, has been reported to be raised in diabetics, the highest concentrations being found in diabetics with complications (155, 156). It is not settled whether this is a primary or secondary phenomenon.

Lysosomal enzyme. In studies on cultured human skin fibroblast we have demonstrated an increased release of β -hexosaminidase by increasing glucose concentrations. Non-diabetic cells did not

differ from type 2 diabetics. No change in intracellular lysosomal enzyme activity was noted. Thus it is proposed that increased plasma levels of lysosomal hydrolases from human diabetics might be due to increased release induced by glucose. Experiments are in progress to further evaluate this finding.

Cell cultures. These studies illustrate a possible experimental approach to evaluate pathophysiological factors in diabetic complications by cultured cells from affected subjects. It is evident that caution should be exercised in the extrapolation of observations from cell culture studies to the in vivo situation.

This model system is also useful in evaluating drug effects (157) or drug toxicity (158). Comparative studies on human endothelial and human arterial smooth muscle cells are also used at our laboratory.

E. GENERAL SUMMARY

A cell culture system of human skin fibroblasts was elaborated and used in biosynthetic studies of collagen, total protein, glycosaminoglycans, and lysosomal enzyme. The influence of insulin and glucose levels was specially studied because of the importance of these factors for diabetic vascular complications.

Heparin was demonstrated to increase collagen and total protein production in non-diabetic, type 1 and type 2 diabetic cells.

Glucose was found to increase the release of β -hexosaminidase similarly in fibroblasts from non-diabetic and type 2 diabetic subjects.

Collagen production was found to be enhanced by insulin in a dose-response relationship within physiological insulin levels. Fibroblasts from diabetic donors appeared to be more responsive at low insulin levels. Collagen production was also demonstrated to be stimulated by platelet derived growth factor(s). Glucose increased collagen production in fibroblasts from non-diabetics and type 1 diabetics. The latter was different to fibroblasts from type 2 diabetics which were unaffected by glucose concentrations.

This study has shown that the cultured fibroblast is influenced by environmental factors important to diabetes mellitus. In addition, some differences have been found between fibroblasts from non-diabetic, type 1 diabetic and type 2 diabetic donors. The findings may be of relevance for the pathophysiology of complications in diabetes mellitus.

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The analysis of β -hexosaminidase was performed at the Department of Clinical Chemistry, University of Lund, Lund, Sweden.

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STIMULATION OF COLLAGEN PRODUCTION IN GROWTH-ARRESTED MYOCYTES AND FIBROBLASTS IN CULTURE BY GROWTH FACTOR(S) FROM PLATELETS

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SUMMARY

Human fibroblasts, rabbit aortic fibroblasts and rabbit aortic myocytes were growth-arrested in serum-free media and stimulated with material spontaneously released from human platelets in vitro. The cells increased their collagen production 2- to 3-fold with increasing platelet factor concentration, whereas the cell mass, measured by total DNA, was unaffected. An increase in [³H]thymidine incorporation into DNA was noted, however. Thus, platelets can release factors stimulating collagen synthesis, without necessarily inducing concomitant cell division.

Proliferation of arterial smooth muscle cells (SMC) is a key event in the development of atherosclerosis [1]. In more advanced stages of this disease increased deposition of collagen is a constant feature [1]. This collagen is synthesized by SMC or fibroblasts but the knowledge of regulating factors is very limited [2, 3].

The platelet-derived growth factor (PDGF) is released from platelet alpha-granules during aggregation [4] and in arterial SMC stimulates proliferation, uptake and storage of lipids, and to an increased synthesis of connective tissue material, such as collagen [5]. Connective tissue-activating peptide III (CTAP-III) is similar to PDGF. CTAP-III is also isolated from human platelets and can increase collagen synthesis in human connective tissue cells [6].

It is not settled whether cell proliferation is necessary for the platelet factors to cause an increased collagen synthesis. Burke & Ross consider the increased collagen syn-

thesis to be an expression of cellular proliferation [7]. In contrast, Breul et al. conclude from their work with a strain of human embryonic lung fibroblasts that collagen production is held remarkably constant, independent of the proliferative state [8].

We have studied the effect of material spontaneously released from platelets on collagen production in confluent SMC and fibroblasts in culture, maintained in serum-free media to minimize proliferation.

MATERIAL AND METHODS

Cell culture

Rabbit SMC were grown from explants of medial segments of the thoracic aorta from normal rabbits as described by Ledet [9].

Fibroblasts from rabbit aorta were obtained from adventitial fragments collected from corresponding vessel segments.

Human fibroblast cultures were established from 3 mm skin punch biopsies taken, with informed consent, from the medical aspect of the left arm in healthy subjects.

All cell types were cultured under similar condi-

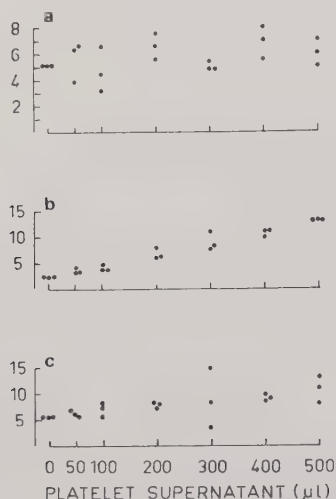


Fig. 1. Effects of platelet supernatant on (a) DNA mass (μg); (b) $[^3\text{H}]$ thymidine incorporation ($\text{cpm} \times 10^{-4}$); (c) collagen production ($\text{cpm}/\mu\text{g DNA} \times 10^{-3}$) in rabbit aortic fibroblasts.

tions. 8–10 explants were incubated in 24 cm^2 plastic flasks (Nunc A/S) containing 2 ml of Minimum Essential Medium (MEM) (Gibco), Hepes buffered (20 mM), supplemented with 10% fetal bovine serum (Gibco heat-inactivated), L-glutamine 585 mg/l, non-essential amino acids (NEAA) 1%, and gentamicin 0.1 mg/ml. The flasks were incubated at 37°C and left untouched for 10 days to establish adherence and primary growth. Growth was observed from approx. 80% of the explants after 7 days for rabbit vessel wall cells and 2–3 weeks for human fibroblasts. Medium was changed twice a week. Subcultures were obtained by using 0.05% trypsin, 0.02% EDTA. Confluent cells from passages 2–4 were used for the experiments.

Experimental procedure

Three ml of a suspension of trypsinized cells was transferred to 10 cm^2 plastic Petri dishes (Nunc) and grown to confluency. The cell layer was washed twice with 2 ml Dulbecco phosphate-buffered saline (PBS) (Flow) and 3 ml serum-free MEM, containing the above-mentioned supplements, was added. The cells were left for 2 days under these conditions to attain growth arrest.

Growth-stimulating material was obtained by isolating platelets from healthy human subjects as described by Mattiasson et al. [10]. Blood was collected in 10 ml siliconized tubes, each containing 1 ml NaCl 0.12 M and EDTA 0.027 M. The platelet-rich plasma obtained contained approx. 3×10^{11} platelets/ml. Platelets were isolated by centrifugation at 6500 g for 10 min and then resuspended in equivalent amounts of Krebs-Ringer bicarbonate buffer without Ca^{2+} and Mg^{2+} . After spontaneous efflux of platelet material in this

buffer for 120 min at 37°C the supernatant was used for the experiments. This supernatant contained 400–600 μg protein/ml.

To the growth-arrested cells in serum-free media 0, 50, 100, 200, 300, 400, or 500 μl of platelet supernatant was added (day 3), and incubation was continued for an additional 48 h. Three dishes were taken for DNA measurements when incubation with platelet supernatant was started.

DNA determination

Cells were scraped with a plastic policeman from each dish, and DNA was determined by a diamino-benzoic acid fluorometric method as described by Fujimoto et al. [11].

Cell counting

This was performed in a Bürker hemocytometer chamber.

Collagen determination

For collagen analysis 48 h labelling with $[^3\text{H}]$ proline (20 $\mu\text{Ci}/\text{ml}$) (Radiochemical Centre, Amersham) was used. The $[^3\text{H}]$ proline-labelled collagenase-digestible protein in the medium was used as a measurement of collagen secretion according to the method described by Peterkofsky & Diegelmann [12]. With each sample, correction was applied by subtracting counts obtained with aliquots processed without collagenase.

Total protein synthesis

Total protein was determined, as part of the above procedures, by measuring $[^3\text{H}]$ proline radioactivity incorporated in trichloroacetic acid (TCA) precipitates of media aliquots untreated with collagenase.

Radioactivity measurements

Collagen and total protein ^3H activities were counted in Biofluor (New England Nuclear) on a Packard B 2450 liquid scintillation spectrometer.

Assay for DNA synthesis

One $\mu\text{Ci}/\text{ml}$ medium of $[\text{methyl-}^3\text{H}]$ thymidine (Amersham) was added to each dish during 24 h between days 3 and 4. The ^3H activity was determined as described by Witte et al. [13] but with 10 ml Aqualos 2 (New England Nuclear) as scintillation liquid.

For each level of platelet supernatant added, three dishes were taken for DNA and collagen analysis and three dishes for determination of $[^3\text{H}]$ thymidine incorporation.

RESULTS

Experiments were repeated three times for each cell type, using explants from different

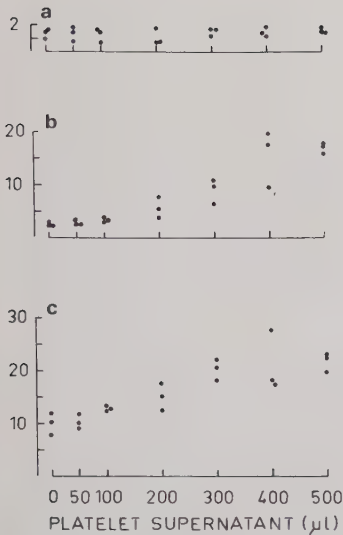


Fig. 2. Effects of platelet supernatant on (a) DNA mass (μg); (b) $[^3\text{H}]$ thymidine incorporation ($\text{cpm} \times 10^{-3}$); (c) collagen production ($\text{cpm}/\mu\text{g DNA} \times 10^{-2}$) in human fibroblasts.

human and rabbit donors, and different platelet supernatant preparations. The results obtained were consistent. In the following, data from one experiment with each cell type will be presented.

Rabbit aortic fibroblasts

At the start of experimental incubation the dishes contained about 0.40×10^6 cells or $5.0 \mu\text{g DNA}$. Maximal $[^3\text{H}]$ thymidine incorporation was 13 300 cpm. No significant change in DNA was noted with increasing amounts of platelet releasate, and at the end of incubation, the dishes contained approximately the same amount of cells (0.35×10^6).

Addition of platelet supernatant caused an increase of collagen production (radioactivity incorporated per $\mu\text{g DNA}$) which was dose-dependent and reached about 2-fold values with $500 \mu\text{l}$ added to 3 ml medium (fig. 1). The $[^3\text{H}]$ thymidine incorporation was also increased.

The correlation between parameters measured and supernatant concentrations was determined. There was no correlation ($r=0.37$) for DNA measurements. Collagen production was significantly ($r=0.69$, $p<0.001$) increased with increasing concentration of platelet supernatant. Similar results were found for $[^3\text{H}]$ thymidine incorporation ($r=0.98$, $p<0.001$). Student's unpaired *t*-test confirmed significant effects, when comparing the two highest with the two lowest platelets supernatant concentrations.

Human skin fibroblasts

These cells grew to a lower density than rabbit fibroblasts, the dishes containing about 0.15×10^6 cells or $1.6 \mu\text{g DNA}$. As shown in fig. 2, collagen production, both in total and relative amounts, was likewise considerably lower in the human cells.

With increasing concentrations of platelet supernatant essentially the same results as for rabbit fibroblasts were obtained, i.e., a 2-fold increase in collagen production ($r=0.87$, $p<0.001$), unaffected total DNA mass and an increase in $[^3\text{H}]$ thymidine uptake ($r=0.93$, $p<0.001$).

Rabbit aortic SMC

Cell density was 0.45×10^6 cells/dish, or $3.5 \mu\text{g DNA}$.

A representative experiment is shown in fig. 3. Similar results were obtained with these cells, i.e. no change in DNA mass, but significant correlation between collagen production and increasing platelet supernatant concentration ($r=0.90$, $p<0.001$) as well as for $[^3\text{H}]$ thymidine incorporation ($r=0.69$, $p<0.001$).

Total protein synthesis

Collagen production was 35–40% of total $[^3\text{H}]$ proline incorporation. The proportion

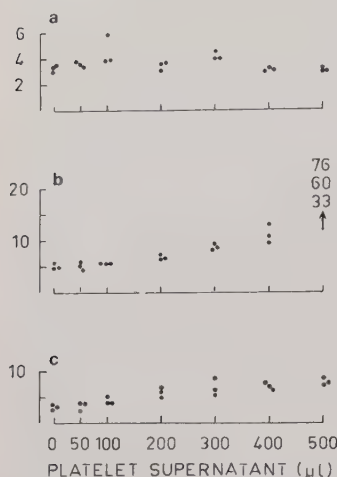


Fig. 3. Effects of platelet supernatant on (a) DNA mass (μ g); (b) [3 H]thymidine incorporation (cpm $\times 10^{-2}$); (c) collagen production (cpm/ μ g DNA 10^{-3}) in rabbit aortic myocytes.

of total incorporation recovered as collagen was similar in all experiments, independent of cell type and platelet supernatant concentration.

DISCUSSION

Whether platelet factors have a selective effect on collagen synthesis that can be separated from the proliferation stimulating effect is not known. Most studies on this item have been performed with whole serum as stimulant [7, 8].

In order to study only the effect of material released from platelets, the present measurements of collagen synthesis were performed after the cells had reached a growth-arrested state in a serum-free system for two days.

The regulation of the fibroblast cell cycle has been described by Scher et al. [14]. SMC and fibroblasts do not proliferate in the absence of serum. In plasma the cells reach a state called 'quiescence', where they remain alive but do not proliferate.

When PDGF is added to platelet-poor plasma serum the growth-promoting effect corresponding to whole serum is restored. PDGF induces 'competence' in growth-arrested cells to respond to 'progression factors' from platelet-poor plasma, mainly somatomedins. PDGF alone is not able to allow competent cells to enter S phase, and thus DNA synthesis and cell division are almost completely inhibited in serum-free media.

In the present experiments no significant changes in cell DNA or cell number were noted during the incubation period.

When larger amounts of platelet supernatant were added an increased incorporation of [3 H]thymidine was seen but this may not reflect actual cell division and proliferation under the prevailing conditions. Probably, DNA synthesis occurs in a minor part of the cells, but to an extent too small to influence the total DNA mass. This was checked by flow cytofluorometry (Ortho Instruments 50 L cytofluorograph), comparing confluent growth-arrested SMC with cells stimulated with platelet supernatant (500 μ l/3 ml medium). Identical results were obtained, with 94% of the cells in G0/G1 phase, 5% in G2 and 1% in S. In all probability, collagen synthesis occurred predominantly in G0/G1 cells. An undetectably small proportion of cells may have entered or traversed S phase, being responsible for the [3 H]thymidine incorporation.

Fibroblasts and SMC did not differ in their capacity to respond to platelet factors with collagen production.

Our results show that factors from platelets can increase collagen production in non-proliferating cells 2- or 3-fold without a corresponding increase in cell mass. Cell proliferation therefore does not seem necessary for platelet factors to increase collagen production. This could be of significance in

vivo to explain the development of extensive fibrosis when cell proliferation is scanty, e.g. in the aging cell or when cells are 'contact inhibited'.

There is no direct evidence of identity between the factor(s) increasing replication and that (those) increasing collagen production. Whether PDGF alone is responsible or not, there is evidence [10, 15] that material spontaneously released from platelets, as used in the present study, contains growth-stimulating activity and beta-thromboglobulin, but no dense body components.

Determination of the identity of the factor(s) involved in stimulation of collagen synthesis requires the application of fractionation procedures to the mixture of alpha granule contents spontaneously released by platelets or to material obtained from isolated alpha granules. Such studies are in progress.

The present results warrant the conclusion that factors spontaneously released by human platelets in vitro stimulate collagen synthesis without necessarily inducing concomitant cell proliferation.

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EFFECTS OF HEPARIN AND DEXTRAN SULPHATE ON THE PRODUCTION OF COLLAGEN AND PROTEIN IN DIABETIC AND NON-DIABETIC HUMAN SKIN FIBROBLAST CULTURES

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ABSTRACT

Addition of heparin and dextran sulphate to human skin fibroblasts in cell cultures caused an increase in [^3H]-proline incorporation into collagen and total protein in the culture medium by cells derived from non-diabetics. Cells from type 2 diabetic subjects were significantly less affected by dextran sulphate addition, suggesting altered regulatory mechanisms for collagen production in these cells.

Addition of chondroitin sulphate caused a dose-dependent increase in labelled collagen, indicating a possible role for this glycosaminoglycan as modulator of collagen deposition.

KEY WORDS: HEPARIN; DEXTRAN SULPHATE; COLLAGEN DIABETES MELLITUS; FIBROBLASTS

INTRODUCTION

Collagen and glycosaminoglycans are essential components of connective tissue. They are also of major interest in the pathophysiology of atherosclerotic and diabetic vessel wall lesions as well as in basement membrane changes. In diabetes mellitus, collagen-rich basement membranes are thickened, and an increased incidence of degenerative arterial wall lesions is characteristic.

The regulatory mechanisms in the formation and deposition of extracellular matrix are largely unknown. In recent years the role of fibronectin has become apparent. Fibronectin binds to collagen and mediates the attachment of cells to collagen; it has also been found to interact with heparin and dextran sulphate (10, 14). Such interactions between connective tissue components may regulate the synthesis and/or deposition of these materials. Furthermore, it has been claimed (4, 5, 12) that skin fibroblasts from diabetics display metabolic abnormalities, supposedly related to their diabetic genotype. Studies employing fibroblast culture would seem well suited to explore the production of collagen.

In the present study, fibroblasts from normal and diabetic individuals were used to examine the possible influence of added polysaccharides on collagen production. Heparin was compared with the artificial homologue

dextran sulphate, and additional studies were made with chondroitin sulphate as representing naturally occurring connective tissue glycosaminoglycans.

MATERIAL AND METHODS

Skin punch biopsies were obtained, after informed consent, from the medial aspect of the left upper arm in nine healthy male subjects, aged 35–53 (Table 1). Biopsies were also obtained from six type 2 (insulin-independent) diabetics, all on sulfonylurea medication, mainly glipizide (Table 2). Three type 1 (insulin-dependent) diabetics on insulin treatment were also included in the study (Table 2).

Cell cultures were established (15) and maintained in Minimum essential medium with Hanks' salts and 25mM HEPES buffer, supplemented with 10 % fetal calf serum, heat-inactivated, 4mM L-glutamine, 1 % non-essential amino acids and 0.1 mg/ml gentamicin (all from Gibco, Chemoferm AB, Sweden).

Cells were subcultured by a gentle trypsinizing procedure (0.025 % trypsin-EDTA, Gibco) and were generally used in early passages, as indicated in the tables, for experiments.

In the experiments, performed in 9 cm² plastic petri dishes (A/S NUNC), the above medium was supplemented with 0.1 mg/ml (unless otherwise stated) dextran sulphate, sodium salt (Pharmacia Fine Chemicals, Sweden), heparin (porcine intestine, AB Vitrum, Sweden) or chondroitin sulphate (whale and shark cartilage, Sigma). The cells were exposed to 2 ml of these media, or to control medium without additives, for 48 hours in the presence of 5-[^3H]-proline (Radiochemical Centre, Amersham, UK), 20 $\mu\text{Ci/ml}$. Tracer incorporation into medium collagen and total protein was measured by the method of Peterkofsky and Diegelmann (9). DNA was determined in

TABLE 1
Fibroblasts from non-diabetics: donor data and [^3H]-proline incorporation into collagen.

Subj.	Age	Sex	P^*	Cpm/ μg DNA**		
				Control	Heparin	Dextran sulphate
1	50	M	2	1000 $\pm$ 234 (3)	1517 $\pm$ 92 (4)	1499 $\pm$ 92 (2)
2	49	M	3	1000 $\pm$ 273 (4)	1195 $\pm$ 467 (2)	1735 $\pm$ 117 (3)
3	54	M	3	1000 $\pm$ 154 (3)	1286 $\pm$ 227 (4)	1076 $\pm$ 244 (2)
4	52	M	2	1000 $\pm$ 163 (3)	1096 $\pm$ 31 (3)	1623 $\pm$ 335 (3)
5	35	M	5	1000 $\pm$ 53 (4)	1296 $\pm$ 105 (2)	1189 $\pm$ 34 (4)
6	47	M	12	1000 $\pm$ 184 (4)	1489 $\pm$ 160 (4)	1028 $\pm$ 58 (3)
7	41	M	3	1000 $\pm$ 145 (2)	2067 $\pm$ 160 (4)	1762 $\pm$ 153 (4)
8	52	M	4	1000 $\pm$ 113 (3)	1716 $\pm$ 125 (4)	1279 $\pm$ 86 (3)
9	51	M	2	1000 $\pm$ 99 (3)	1553 $\pm$ 132 (4)	1239 $\pm$ 84 (4)
$\bar{x}$	47.9		4	1000 $\pm$ 158	1468 $\pm$ 167	1381 $\pm$ 134

* Cell passage number.

** Data are means $\pm$ SEM, with number of culture dishes in brackets.

each dish by a diaminobenzoic acid fluorometric method (3). DNA values were used as measurements of cell mass, and radioactivities of collagen and total protein in the medium were expressed as counts per minute (CPM) per μg DNA.

Calculations. The control dishes of each individual experiment and cell line were standardized to a mean of 1000 CPM/ μg DNA, and the data from the experimental dishes were adjusted accordingly. This procedure made it possible to evaluate statistically the effect of added polysaccharides despite the unavoidable interexperimental variations in CPM/ μg DNA values. There were no statistically significant differences in control CPM/ μg DNA between the three cell donor groups. Results from individual subjects are presented in Table 1 and 2.

Student's unpaired t-test was performed on data from individual dishes pooled into non-diabetic, type 2

diabetic, and type 1 diabetic groups for each experimental condition. Two-tailed p values are shown in the figures ($***P < 0.001$, $**P < 0.01$ and $*P < 0.05$), with the number of dishes given as n . Statistical analysis with the non-parametric Mann-Whitney test gave identical results in terms of significant differences.

RESULTS

In preliminary experiments we noted that adding heparin affected collagen production in cultured rabbit aortic smooth muscle cells. These were prepared and cultured under standard conditions (15). A highly significant

TABLE 2
Fibroblasts from diabetics: donor data and [^3H]-proline incorporation into collagen.

Subj.	Age	Sex	P*	Cpm/ μ g DNA**		
				Control	Heparin	Dextran sulphate
Type 2 diabetics						
1	47	M	5	1000 $\pm$ 137 (3)	1187 $\pm$ 65 (4)	929 $\pm$ 80 (4)
2	46	M	9	1000 $\pm$ 99 (4)	1592 $\pm$ 40 (4)	878 $\pm$ 164 (4)
3	65	M	2	1000 $\pm$ 152 (4)	1836 $\pm$ 64 (2)	1338 $\pm$ 143 (3)
4	51	F	2	1000 $\pm$ 57 (4)	1843 $\pm$ 179 (3)	1892 $\pm$ 234 (2)
5	50	F	2	1000 $\pm$ 39 (4)	1267 $\pm$ 62 (4)	876 $\pm$ 28 (3)
6	52	M	3	1000 $\pm$ 58 (4)	1525 $\pm$ 125 (3)	1033 $\pm$ 135 (3)
$\bar{x}$	51.8		4	1000 $\pm$ 90	1542 $\pm$ 89	1158 $\pm$ 131
Type 1 diabetics						
1	48	M	7	1000 $\pm$ 122 (4)	1547 $\pm$ 264 (4)	1062 $\pm$ 89 (3)
2	67	M	4	1000 $\pm$ 159 (2)	1763 $\pm$ (1)	1355 $\pm$ 112 (3)
3	50	M	6	1000 $\pm$ 30 (3)	—	1750 $\pm$ 89 (2)
$\bar{x}$	55.0		6	1000 $\pm$ 104	1655 $\pm$ 264	1389 $\pm$ 97

* Cell passage number

** Data are means $\pm$ SEM, with number of culture dishes in brackets.

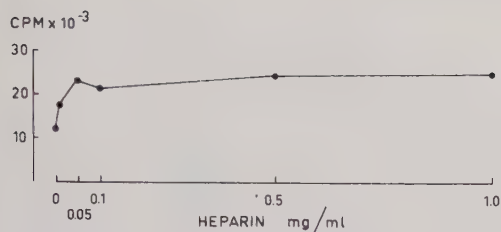


Fig. 1. Effect of heparin on the incorporation of $[^3\text{H}]$ -proline in culture medium collagen by rabbit aortic smooth muscle cells. Labelling period 24 hours. Each point is the mean of 8 dishes.

($2P < 0.001$) increment was obtained in $[^3\text{H}]$ -proline incorporation into collagen in the medium at the lowest heparin concentration, 0.01 mg/ml, with the maximal effect being evident at 0.05 mg/ml (Fig. 1).

Chondroitin sulphate was tested in one normal cell line (N) and in one cell line (D) derived from a type 2 diabetic. Similar numbers of cells (1.8×10^5) were explanted in 8 dishes at every concentration for each cell line, but a difference in cell passage number was chosen (N in passage 5 and D in passage 10) to disclose any major differences due to passage levels. As shown in Fig. 2, the dose-dependent increase of collagen production was parallel in the cell lines.

The time course of medium collagen labelling was studied with normal fibroblasts, 1.5×10^5 cells per dish, without additions or with dextran sulphate or chondroitin sulphate added. As shown in Fig. 3, $[^3\text{H}]$ -collagen steadily increased throughout the experiments. A 48 h labelling period was chosen for further experiments.

In cells from non-diabetic subjects, type 2 diabetics and type 1 diabetics, the presence of heparin caused a 50 % increase in medium $[^3\text{H}]$ -collagen ($P < 0.0001$, $P < 0.0001$ and $P < 0.005$ respectively) as shown in Fig. 4. There was no significant difference between cell groups in the effect of heparin. Adding dextran sulphate had a similar effect only on cells from non-diabetics and type 1 diabetics, significantly different from controls ($P < 0.0001$ and $P < 0.02$). There was no statistically significant difference in these two cell types between the medium collagen increase caused by heparin or dextran sulphate addition. Cells from type 2 diabetics, however, were unaffected by dextran sulphate addition, significantly different ($P < 0.0006$) from the influence of heparin but comparable to controls for this group. The

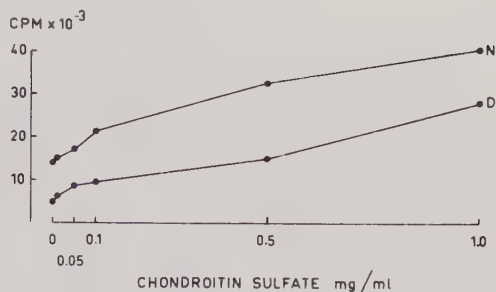


Fig. 2. Effect of chondroitin sulphate addition on the incorporation of $[^3\text{H}]$ -proline in culture medium collagen. Fibroblasts from a normal (N) and a diabetic (D) individual. Labelling period 24 hours. Each point is the mean of 8 dishes.

result of using dextran sulphate on cells from type 2 diabetics were also significantly ($P < 0.008$) different from those of non-diabetics but not significantly different from the few cell dishes obtained from type 1 diabetics.

The effects of the polysaccharides on $[^3\text{H}]$ -proline incorporation into total protein in the medium (Fig. 5) paralleled those on collagen measurements, although the increase caused by heparin addition was minor for all cell types. The lack of response when adding dextran sulphate to cells from type 2 diabetics was still a notable feature.

DISCUSSION

The general effect of the inclusion of polysaccharides in the culture medium was an increase in the amount of $[^3\text{H}]$ -proline radioactivity recovered in collagen and total protein in the culture medium. Given the tracer used, the effects on collagen would be the most important. The polysaccharides may influence synthesis and/or secretion of collagen. In a study of cultured pig arterial endothelial cells it was shown that chondroitin sulphate enhanced the collagen synthesis within the cells, but not the rate of excretion into the medium. Although both chondroitin sulphate and hyaluronic acid were found to be transported to the interior of the cells, only the formed had any effect on collagen synthesis (1). Alternatively, the increase in labelled collagen recovered may be the result of the inhibition of extracellular breakdown (8). The ability of heparin and dextran sulphate to enhance binding of collagen to fibronectin (10, 11) may be a relevant mechanism.

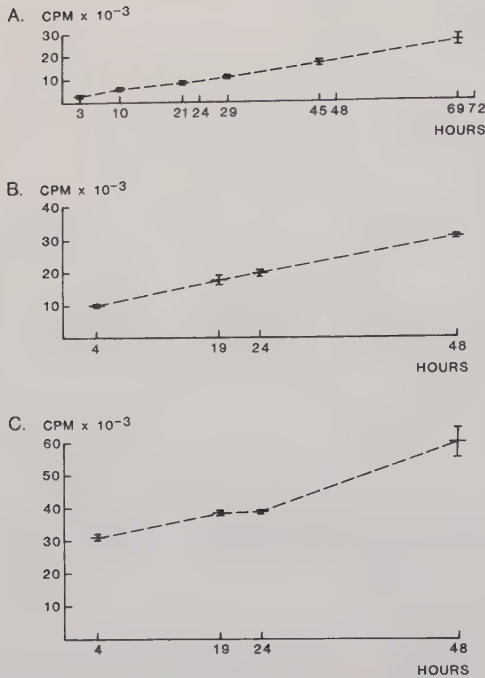


Fig. 3. Time course of accumulation of $[^3\text{H}]$ -collagen in fibroblast culture medium. A) no additions, B) dextran sulphate 0.1 mg/ml, C) chondroitin sulphate 0.1 mg/ml. Means $\pm$ SEM ($n = 8$) are indicated.

The direct effect of heparin on collagen metabolism was noted by Ivaska (6), who reported a decrease of collagen in the medium of isolated rat granuloma cells after exposure to various concentrations of heparin. The mechanism proposed for the interaction between the cell surface and extracellular heparin was that the negative charges of the highly charged heparin bind to positively charged sites in the cell surface and interconnect them, resulting in a restriction of lateral movements in the cell membrane. This proposal implied that the glycosaminoglycan effects resulted from their general polymer and polyanion properties, and the similarity in effects between dextran sulphate and heparin was taken as supporting evidence. In the present study, however, cells from type 2 diabetics behaved differently.

In the present investigation, medium collagen increased in response to heparin, dextran sulphate and chondroitin sulphate additions rather than decreased, the latter finding having been reported by Ivaska (6). The difference in species as well as differences be-

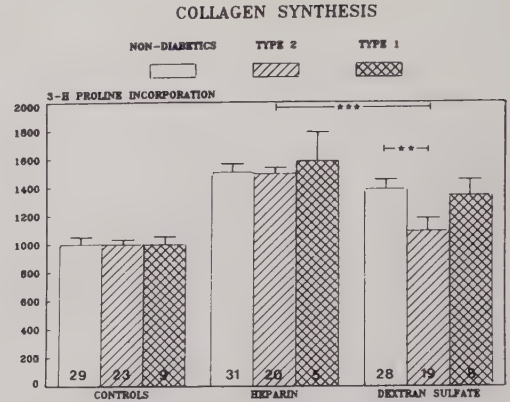


Fig. 4. Effects of heparin and dextran sulphate on $[^3\text{H}]$ -proline incorporation (CPM/ μg DNA) into culture medium collagen by fibroblasts from 9 non-diabetics, 6 type 2 diabetics, and 3 type 1 diabetics. Means $\pm$ SEM. Number of dishes indicated on each bar.

tween isolated and cultured cells could be responsible for the divergent results. Our results are supported by a recent study on human embryonic fibroblasts in culture showing a marked increase in both cell layer (82 %) and medium (177 %) collagen when dextran sulphate (100 $\mu\text{g}/\text{ml}$) was added to serum-free medium (2).

The finding that cells from type 2 diabetics differed from cells from non-diabetics by being uninfluenced by dextran sulphate is notable. At present it is not known whether this difference is related to intracellular or extracellular events. It suggests an altered production of intercellular matrix by cultured

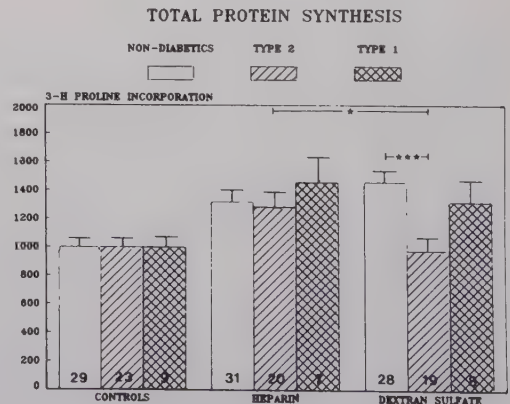


Fig. 5. Effects of heparin and dextran sulphate on $[^3\text{H}]$ -proline incorporation in medium total protein. Conditions as in Fig. 4.

fibroblast in diabetes mellitus, as has been noted for other components (12, 13).

The findings suggest that the extracellular deposition of the three fibroblast secretory products, collagen, fibronectin, and glycosaminoglycans is interdependent. This interaction, and its possible disturbance in diabetics, may be of importance for the quantities and composition of connective tissue produced in normal and pathological conditions (7).

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Insulin Effects on Collagen and Protein Production in Cultured Human Skin Fibroblasts from Diabetic and Non-Diabetic Subjects

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Summary

The influence of insulin on collagen and total protein production was studied in cultured human skin fibroblasts from patients with Type 1 (insulin-dependent) diabetes and Type 2 (non-insulin-dependent) diabetes, and from non-diabetic subjects. Fibroblast cultures were exposed to various concentrations of insulin (0–10⁵ mU/l) for 48 hours in serum-free medium containing tritium labelled proline.

With medium glucose at 5.6 mmol/l, cells from diabetic subjects displayed an increase in collagen and protein radioactivities in the extracellular medium in the presence of insulin at 10² or 10³ mU/l. Concentrations above 10³ mU/l did not stimulate further. In cells from non-diabetic subjects, stimulated incorporation was evident only at insulin 10³ mU/l.

In the presence of high glucose concentration (38.9 mmol/l) in the medium, the lowest insulin level (10² mU/l) did not influence collagen production in any cell type, and the effects of insulin on total protein radioactivity was reduced in all cells.

The enhancement by insulin of collagen secretion, and the higher sensitivity of cells from diabetics to this insulin action, is of interest in relation to the possible role of insulin in the accelerated atherosclerosis observed in diabetics.

Key-Words: Diabetes Mellitus — Human Skin Fibroblasts — Insulin — Collagen — Protein — Proline Incorporation

Introduction

The demonstration of a limited replicative life-span of cultured fibroblasts by Hayslick (1965), and reports on diminished plating efficiency in cultured fibroblasts from diabetic subjects by Goldstein, Littlefield and Soeldner (1969), and a lower than normal replicative capacity of diabetic fibroblasts (Vracko and Benditt 1975; Goldstein, Moerman, Soeldner, Gleason and Barnett 1979) have been followed by conflicting data on the effect of donor age on in vitro life-span of diabetic and non-diabetic fibroblasts (Goldstein, Moerman, Soeldner, Gleason and Barnett 1978; Gleason and Goldstein 1978; Goldstein, Moerman, Soeldner, Gleason and Barnett 1979; Vracko and McFarland 1980). The data suggest a manifestation of senescence at the cellular level, and a possible inherent defect in diabetic cells, although there was no difference in viability between cells from insulin-dependent diabetics and normals in one study by Rosenbloom and Rosenbloom (1978).

Biochemical deviations have also been reported for cultured fibroblasts from diabetics compared with cells from normals. Rowe, Starmann, Fujimoto and Williams (1977) found a higher collagen synthesis in cultured skin fibroblasts from insulin-dependent diabetics than in cells from normal subjects. With regard to glycosaminoglycans, it has been claimed that skin fibroblasts from diabetics (insulin-dependent as well as non-insulin-dependent) produce an increased proportion of heparan sulfate (Silbert and Kleinmann 1979).

Insulin receptors have been studied in cultures of human skin fibroblasts (Rechler and Podskalny 1976), as well as effects of insulin on conversion of ¹⁴C-glucose to ¹⁴CO₂ (Goldstein and Littlefield 1969), glucose uptake, incorporation of leucine into protein, RNA synthesis (Fujimoto, Teague and Williams 1974), and insulin stimulation of glycogen synthase activity (Craig, Huang and Lerner 1980). In the last mentioned process, a difference between cells from normal subjects and cells from diabetics was claimed.

Stout (1979) has suggested an important role for insulin in the development of atherosclerosis in diabetes. This is particularly relevant considering the observations of hyperinsulinaemia in diabetics with atherosclerosis (Santen, Willis and Fajans 1972; Kashyap, Magill, Rojas and Hoffman 1970). The aim of the present study was to elucidate whether proline incorporation into collagen and total protein by cultured skin fibroblasts is influenced by insulin, and whether cells from diabetic patients differ from cells from non-diabetic subjects.

Material and Methods

Skin punch biopsies were taken without anaesthesia, after informed consent had been obtained, from five non-diabetic male subjects aged 48–54 years (mean 50.2), without diabetes among their first degree relatives, and from five male Type 1 diabetics aged 26–49 years (mean 33.6) with ketoacidosis-prone hyperglycaemia and acute onset of symptoms, and also from seven Type 2 diabetics (6 males, 1 female) aged 44–71 years (mean 58.3) without ketoacidosis and with gradual onset of diabetes after the age of 30. The Type 2 diabetics were treated with diet plus oral agents, except for one patient who, although not insulin-dependent, was on insulin treatment. The biopsies were taken from the skin, of the medial aspect of the left upper arm, and then cut into small pieces, washed in culture medium and placed at the bottom in 25 cm² plastic culture vessels (A/S Nunc, Denmark). The vessels were turned upside down and 5 ml medium was added. The biopsies were thus left uncovered with medium for 1–1.5 hours to ensure adhesion before the vessel was turned again to be left untouched during 10 days at 37°C for outgrowth. All medium components were obtained from Gibco. Minimum essential medium with Hanks' salts was supplemented

with fetal calf serum (10%), L-glutamine (585 mg/l), non-essential amino acid mixture (1%) and gentamycin (100 mg/l). During outgrowth, medium was changed twice a week. Similar medium and additions were used for experiments (performed in 9 cm² plastic petri dishes) except for the addition of ascorbic acid (50 mg/l) and omission of serum, if not otherwise stated. Porcine monocomponent insulin (Actrapid, Novo, Denmark) was used at 10^2 – 10^5 mU/l. In some experiments medium glucose concentration was 38.9 mmol/l instead of the ordinary 5.6 mmol/l.

The cells were used in passages 2–12, in all cases but one non-diabetic passages 2–8, without differences between the groups. In subculturing a 1:2 split ratio was used; thus, one passage = one population doubling. A gentle trypsinization with 0.05% trypsin – 0.02% EDTA was used for subculturing. The cells were grown to confluency and then transferred to experimental dishes with trypsinization 24 hours before the start of experiments, which were performed at sub-confluent cell densities. In control experiments there was no change in DNA mass per dish before and after experimental variations used in this study and, although not specifically examined, no growth differences between the cell lines were noticed. L-(5-³H) proline (Radiochemical Centre, Amersham, U.K.) was added to the 2 ml medium in experimental dishes (25 µCi/ml). The labelling period was 48 hours. Radioactive proline incorporation in collagen and total protein in the medium were measured by the collagenase method of *Peterkofsky and Diegelmann* (1971). The DNA content of each experimental dish was measured by a fluorometric method (*Fujimoto, Teague and Williams* 1977). Experimental data are expressed as cpm/µg DNA.

Statistical analysis was performed after normalization of data to a mean of 1000 cpm for the control dishes in each experiment. The results of individual dishes (n) were considered a sample of cells from non-diabetics, Type 1, and Type 2 diabetics. The number of donor subjects for every experimental variation is given in figure legends. Student's unpaired t-test was used, where not otherwise stated, and p values represent two-tailed significance levels.

Results

An increased proline incorporation into both collagen and total protein in the medium was noted in all cell cultures when insulin was added to serum-free medium. No changes in DNA mass per dish was noticed during the incubation period in any cell type.

Fig. 1 shows a significant increase, with cells from non-diabetic subjects, in radioactive collagen in the medium at an insulin concentration of 10^3 mU/l. Higher insulin levels did not further significantly increase collagen radioactivity. Linear regression analysis of the relationship between proline incorporation (means for each cell donor) and insulin levels gave a correlation coefficient of 0.97 ($P < 0.01$). The incorporation of ³H-proline into total protein was similarly insulin-dependent.

When 10% fetal bovine serum was present during experiments, the effects of insulin were totally abolished. In fact, with high insulin levels (10^5 mU/l) collagen and total protein radioactivities in the medium were somewhat suppressed.

Fig. 2 shows the results with cells from type 1 diabetics. These cells also present an insulin stimulation of collagen production, evident in this case at the lowest insulin concentration (10^2 mU/l) and increasing at 10^3 mU/l. The incorporation means from each donor were highly correlated to insulin levels in the range 0 – 10^2 – 10^3 mU/l ($r = 1.0$). Further increase of insulin to 10^4 mU/l gave a reduction in collagen radioactivity. The same response pattern was

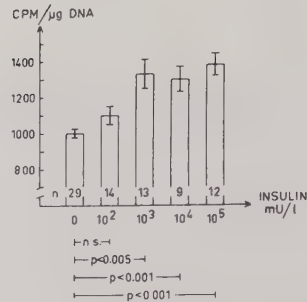


Fig. 1 Skin fibroblasts from non-diabetic subjects. Insulin influence on collagen radioactivity in the medium ($M \pm SEM$). Medium glucose 5.6 mmol/l. n = number of dishes from five subjects at 0, three at 10^2 , three at 10^3 , two at 10^4 and two at 10^5 mU/l insulin.

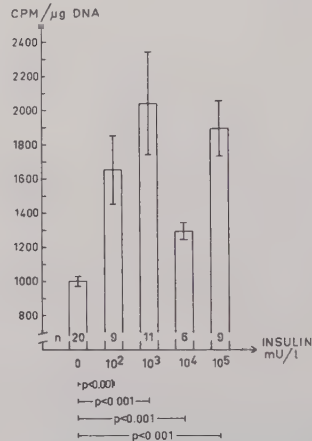


Fig. 2 Skin fibroblasts from insulin-dependent diabetics. n = number of dishes from five subjects at 0, three at 10^2 , four at 10^3 , two at 10^4 and three at 10^5 mU/l insulin. Otherwise as in Fig. 1.

seen with regard to total protein production. The cells studied at 10^4 mU/l are derived from only two subjects. However, the same reduction of collagen radioactivity was observed at this insulin level in cells from the three subjects with non-insulin dependent diabetes (Fig. 3), and thus cells from both types of diabetics differ at this point from cells from non-diabetic subjects.

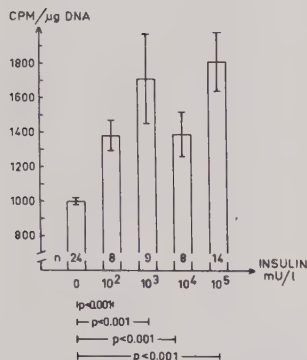


Fig. 3 Skin fibroblasts from non-insulin-dependent diabetics. n = number of dishes from seven subjects at 0, three at 10^2 , three at 10^3 , three at 10^4 and four at 10^5 mU/l insulin. Otherwise as in Fig. 1.

With cells from type 2 diabetics the insulin effects on collagen (Fig. 3) and total protein radioactivities were similar to those on cells from Type 1 diabetics.

Thus fibroblasts from both groups of diabetic subjects showed an increase in medium radioactivities in collagen and total protein at the lowest insulin level used, 10^2 mU/l. At this insulin concentration, there were no significant effects on cells from non-diabetic subjects. Group comparisons with cells from non-diabetics revealed that proline incorporations were higher in cells from Type 1 diabetics ($P < 0.005$) as well as in cells from Type 2 diabetics ($P < 0.01$). With insulin at 10^3 mU/l, the difference from non-diabetic cells was still observed in cells from Type 1 diabetics ($P < 0.025$).

For further statistical evaluation collagen radioactivity in the medium was compared without insulin addition, in a series of eight experiments. In each experiment cells from different individuals (five non-diabetic subjects, six Type 1 and six Type 2 diabetics) were run simultaneously. Wilcoxon's matched pairs signed rank test was used for these calculations. There were no significant differences between fibroblasts from Type 1 and Type 2 diabetics, or between cells from non-diabetic subjects and Type 1 diabetic patients. Cells from non-diabetic subjects, however, had a higher collagen production per μg DNA than cells from Type 2 diabetics ($P < 0.05$).

The effect of insulin on collagen and total protein radioactivity in the medium was also studied with high glucose (38.9 mmol/l) medium. Other medium components and experimental conditions were unchanged. Cells from one non-diabetic subject, one Type 1 and one Type 2 diabetic were used. The high glucose medium had no general influence on insulin effects on collagen production compared to experiments with normal glucose. The drop in collagen production at the 10^4 mU/l insulin level, observed at 5.6 mmol/l glucose,

remained in the fibroblasts from the insulin-dependent subject at high glucose. The cells from one non-insulin dependent subject maintained a high collagen production at the insulin level 10^4 mU/l, in contrast to the drop noticed in the same cell line at 5.6 mmol/l medium glucose. The lowered collagen production in Type 1 cells was significantly ($P < 0.05$) different from the Type 2 diabetic cells at 10^4 mU/l of insulin in glucose enriched medium. The total protein radioactivity in the medium displayed a similar reaction to insulin, as collagen, in high glucose medium but the stimulatory effect of insulin was less pronounced. Thus, the increase in total protein radioactivity was significantly raised only at an insulin level of 10^4 mU/l with cells from the Type 2 diabetic and the non-diabetic, but in cells from the Type 1 diabetic there was no significant influence of insulin at all.

Discussion

It is well established that the addition of insulin to cultured cells enhances growth in most cases. However, pharmacological levels of insulin have, as a rule, been needed for this proliferation stimulation (Petrides and Böhlen 1980). The lowest concentrations of insulin used in the present study (10^2 and 10^3 mU/l) are within the range that may be observed for plasma insulin in man. Insulin additions were only made at the start of the 48 hours incubation. Insulin assays on a number of medium samples showed well maintained concentrations as found also by Pfeifle, Ditschuneit and Ditschuneit (1980).

No evidence of increased cell mass (DNA) was seen with any insulin level including 10^5 mU/l. This lack of growth-stimulating effect may be due to the complete absence of serum during incubation.

An increased proline incorporation into collagen in the medium was noted at the lowest insulin level, 10^2 mU/l, with cells from diabetics, whereas cells from non-diabetics were not significantly insulin-stimulated until 10^3 mU/l. Increasing insulin beyond 10^3 mU/l did not stimulate further in any type of cells.

There were some indications that markedly elevated glucose concentration in the medium disturbed insulin effects, mainly on the synthesis of total protein. Further experiments are in progress.

Other studies (Termini, Tavella, Donnelly, Di Ferrante, Hill, Lynn and Hatton 1980; Kohn and Hensse 1977; Hamlin, Kohn and Luschkin 1975) have indicated the diabetic state to be associated with physico-chemical changes in collagen from cultured cells or from connective tissue biopsies. This is of interest for the understanding of the premature atherosclerosis in diabetics. Collagen comprises about 40–60% of the protein in human fibrous atherosclerotic plaques. It is thought that medial smooth muscle cells play an important role by proliferation and deposition of collagen in the subintima. Electron microscopic examination of atherosclerotic plaques by McCullagh and Balian (1975) has revealed cells with the appearance of fibroblasts engaged in active protein synthesis. The premature diabetic atherosclerosis could thus be produced by physico-chemical changes in

collagen as well as by accelerated collagen deposition stimulated by temporary or permanent hyperinsulinaemia, as suggested by this study. This would be compatible with clinical data indicating higher insulin levels in diabetics with atherosclerosis (Santen, Willis and Fajans 1971).

The present study takes into consideration only the collagen and total protein radioactivities recovered in the culture medium. It is recognized that insulin effects and group differences observed may be related to one or more of several processes, i.e., tracer uptake, and the rates of synthesis, secretion and degradation of collagen.

In our study there was a somewhat higher tracer incorporation into collagen in cells from non-diabetics than in cells from non-insulin-dependent diabetics, which is in contrast to the results of Rowe, Starman, Fujimoto and Williams (1977). Their experiments were performed in 10% fetal bovine serum, the cells had been frozen and stored in liquid nitrogen, and the cells were at confluency when studied. Some of these factors could account for the difference in findings.

We found that the recovery of labelled collagen and total protein in the medium was clearly stimulated by insulin. This was evident with all types of cells. However, cells from Type 1 and Type 2 diabetic subjects displayed a higher sensitivity to low insulin levels than cells from non-diabetics. The present studies *in vitro* may have counterparts *in vivo* in the proposed role of collagen in the development of atherosclerosis (Ross and Glomstein 1976), and in the possible relation of elevated insulin levels (spontaneous or treatment-induced) to this process.

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Note added in proof. After the completion of these studies, stimulation by insulin on collagen synthesis in chondrosarcoma cells was reported by Bembek et al., *Biochem. Biophys. Res. Commun.* 106: 338 (1982).

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Influence of Glucose on Collagen and Protein Production in Cultured Human Skin Fibroblasts from Diabetic and Non-Diabetic Subjects

by

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ABSTRACT

The effect of glucose on extracellular production of collagen and protein was studied in cultured human skin fibroblasts. Fibroblasts from eight non-diabetics, eight type 1 and twelve type 2 diabetic subjects were cultured. Incorporation of tritium labelled proline into medium collagen and protein was studied at glucose concentration 1 mg/ml to 7 mg/ml (5.6 mM to 38.9 mM).

A seventy-five per cent increase in collagen production was noted by cells from type 1 diabetics at glucose concentration of 5 mg/ml. A considerably lower although significant increase was also noted at this level by cells from non-diabetics. Glucose had no effect on collagen and minimal on protein production by cells from type 2 diabetic subjects.

The results indicate an effect of glucose on extracellular matrix formation in diabetes mellitus which might be relevant for diabetic angiopathy.

INTRODUCTION

It is generally agreed that treatment of diabetes mellitus should aim at normoglycemia above all. Hereby is implied a normalization of other metabolic abnormalities in diabetes. However, the development of large vessel disease in diabetic patients does not appear to be prevented by available methods of treating diabetes (1).

Human skin fibroblasts in culture is a model system for studying possible inherent cellular defects and the cellular reaction to one stimulus at a time. According to the response to injury

theory of pathogenesis of atherosclerosis (2) the endothelial and smooth muscle cells of the arterial wall would be those most closely associated with the development of atherosclerosis. Human arterial endothelial or smooth muscle cells cannot as yet readily be obtained and cultured from different types of human diabetics and non-diabetics whereas human skin fibroblasts are easily available.

The major metabolic abnormality in diabetes mellitus is a high plasma glucose, therefore the present study concerns the effect of glucose on collagen and protein production by cultured skin fibroblasts from non-diabetics, type 1 and type 2 diabetic patients.

METHODS

Skin punch biopsies, 2-3 mm in diameter, was obtained after informed consent from the medial aspect of the left upper arm. No anesthesia was given. Biopsies from eight non-diabetics (aged 42 ± 17 years), eight type 1 (insulin-dependent; aged 40 ± 17) diabetics and twelve type 2 (insulin-independent; aged 57 ± 10) diabetics were used in the study. Individual details of donors are given in Table 1. All type 1 diabetics were on insulin treatment. Five of the type 2 diabetics were on diet alone while six were on diet and sulphonylurea treatment and one was on diet and insulin treatment (presented topmost in table 1).

Cell cultures were established (3) and maintained in Minimum essential medium with Hanks' salts and 25 mM HEPES buffer, at 37°C in air atmosphere, supplemented with 10 % fetal calf serum, heat inactivated, 4 mM L-glutamine, 1 % non-essential amino acids and 0.1 mg/ml gentamicin (all from Gibco, Chemoferm AB, Sweden). Cells were grown to confluency, from the primary explants, in a 25 cm^2 cell plastic tissue culture flask and then transferred to a 81 cm^2 plastic tissue culture flask (A/S NUNC, Roskilde, Denmark). During outgrowth, medium was changed twice a week. In subculturing a 1:2 split ratio was used; thus one passage equals one population dou-

Table 1. Individual characteristics of donors.

Non-diabetics

Donor	Sex	Age	P
1	M	40	5
2	F	22	5
3	M	48	5;7
4	M	51	9
5	M	56	3
6	M	9	4
7	M	56	7
8	M	52	7

Type 1 diabetics

Donor	Sex	Age	P	Dur	Comp1	Weight	Length	Her
9	M	35	3	15	R+P	75.2	176	+
10	M	24	3	2	0	71.5	196	+
11	M	27	4	1	0	67.9	179	0
12	M	25	3	2	0	61.8	174	0
13	M	76	8	53	N	65.4	172	+
14	M	49	10	2	0	72.6	178	+
15	M	34	3	21	R	96.2	185	0
16	M	51	3	36	N	91.8	181	0

Donor	Sex	Age	P	Dur	Comp1	Weight	Length	Her	Treatment
17	M	63	5	7	0	51.3	164	0	I
18	M	58	3	4	N	89.0	176	+	D
19	M	64	7	1	N	73.3	176	0	S
20	M	61	2;7	1	0	75.8	182	0	D
21	F	67	3	15	N+R	75.6	169	+	S
22	M	65	7	12	N+P	63.6	169	0	S
23	M	56	2	10	0	106	171	+	D
24	F	65	2	11	N	64.5	159	+	S
25	M	63	2	2	N	76.8	175	0	S
26	M	39	2	2	0	95.5	175	+	D
27	M	42	2	9	R	73.4	183	+	S
28	M	47	2	0.5	0	85.7	178	+	D

Age in years; P = Cell passage number at experiment. In two cases were experiments repeated at different passages; Dur = Duration of diabetes mellitus, years; Comp1 = P, proteinuria; N, neuropathy; R, retinopathy; 0, no complications; Body weight in kg; Length in cm; Her = diabetes mellitus among first and/or second degree relatives. +, presence; 0, absence. Treatment = I, insulin (16U); D, diet; S, sulphonylurea

bling. A gentle trypsinization with 0.025% trypsin-EDTA (Gibco) was used for subculturing. The individual passage numbers used in experiments are detailed in table 1.

Experimental design

Each cell line was grown to confluency in the 81 cm² flask. The cell layer was rinsed twice with phosphate buffered saline, Dulbecco's modification (PBS) (Gibco) and 3 ml trypsin solution was added. The cell suspension was transformed into a 50 ml sterile plastic vessel (Falcon) and the trypsin solution was inactivated with 5 ml heat inactivated calf serum and addition of 30 ml culture medium. Two ml samples of the cell solution was distributed into, generally, 10-16 experimental 9 cm² plastic petri dishes (A/S Nunc, Denmark). The cells were not counted at this procedure, as the DNA content of every dish was measured at the end of each experiment. The cells were allowed to settle for 24-48 hours before starting the experiments and at this stage the cell layers had not reached confluency. There were thus differences between the cell lines in the amount of DNA but the cell mass (DNA) were kept within a narrow range for each cell line. Experiments were started after three rinses with 2 ml PBS, and 2 ml fresh medium, as above, was added with the appropriate glucose concentrations adjusted from a sterile stock solution. Ascorbic acid (50 µg/ml) was added to the culture medium to ensure maximal collagen synthesis. 5-(³H)-proline (Radiochemical Centre, Amersham UK), 20 µCi/ml, was also added to each dish. Glucose concentrations in the medium was studied in separate experiments using cells from non-diabetic and type 1 diabetic donors. The concentrations of glucose in the media were checked, using a hexokinase method with photometric reading. Samples were tested at all experimental glucose concentrations (1, 3, 5 and 7 mg/ml) at 0, 10, 24, 48 and 72 hours. The variations in measurements were within 10% of the pre-determined glucose levels and no variations could be detected due

to ascorbic acid addition.

Each cell line was tested at the various glucose concentrations in one individual experiment and analyses were performed simultaneously for the individual experiment. The incubation period with (^3H)-proline was 48 hours.

Analysis

Tracer incorporation into collagenase-digestible protein in the medium was used as a measurement of collagen secretion, according to the method of Peterkofsky and Diegelmann (4). With each sample, correction was applied by subtracting counts obtained with aliquots processed without collagenase. Total protein was determined, as part of the above procedures, by measuring (^3H)-proline radioactivity incorporated in trichloroacetic acid (TCA) precipitates of media aliquots untreated with collagenase. Radioactivity measurements were performed in 10 ml Biofluor (New England Nuclear) on a Packard B 2450 liquid scintillation spectrometer. DNA (deoxyribonucleic acid) was determined in each dish by a diamino-benzoic acid fluorometric method (5). DNA values were used as measurements of cell mass, and radioactivities of collagen and total protein in the medium were expressed as counts per minute (CPM) per μg DNA.

Statistics

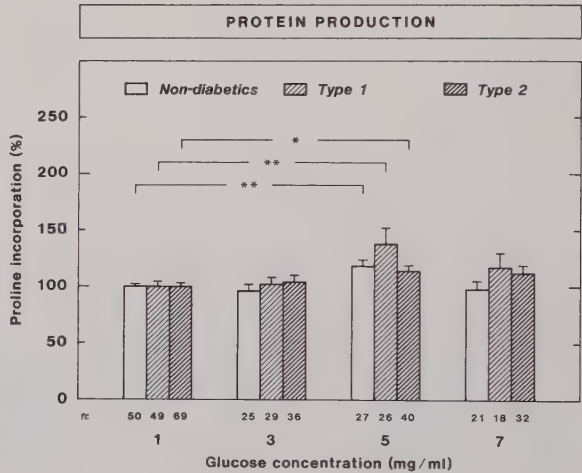
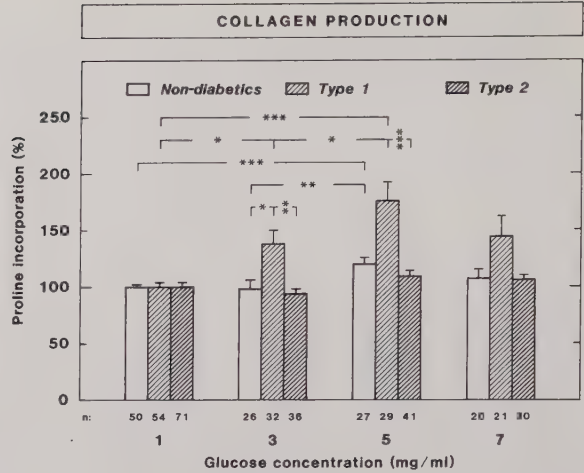
In calculations, the control dishes (glucose 1 mg/ml) of each individual experiment and cell line were standardized to a mean of 100 CPM/ μg DNA, and the data from the remaining dishes were adjusted accordingly. This procedure made it possible to evaluate statistically the effect of glucose additions despite the unavoidable inter-experimental variations in CPM/ μg DNA values.

Non-parametric statistics have been used in all calculations. Kruskal-Wallis analysis of variance was used to test whether the

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LEGENDS

Figure 1. The effect of glucose on (^3H)-proline incorporation into extra-cellular collagen is expressed as % change in relation to a 100% level at 1 mg/ml glucose concentration. n = number of dishes. M $\pm$ SEM.



sets of data have been drawn from the same population. This was performed on data from individual dishes pooled into non-diabetic, type 1 diabetic, and type 2 diabetic groups for each glucose level (figures 1 and 2). The p values (< 0.0001) obtained for collagen production and for protein production (< 0.009) demonstrated significant differences. The Mann-Whitney test was then applied for

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further evaluation between two groups at a time. The p values are shown in the figures (** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$). To reconfirm the results the data were arranged for each glucose level but on an individual donor basis, table 2. The data were then grouped into non-diabetics, type 1 and type 2 diabetics. The Kruskal-Wallis test showed a significant ($p < 0.03$) difference between the groups for glucose effects on collagen production but not for protein production. No further tests were therefore performed regarding protein production.

Osmolarity

The effect of osmolarity on collagen production was examined using three cell lines from non-diabetic donors and two from type 1 diabetics. The glucose was replaced with mannitol at similar concentrations and 1.4×10^5 cells were exposed to (^3H)-proline for 48 hours in plastic wells (4.5 cm^2). Eight wells from each cell line were tested at every concentration. In contrast to the results with glucose addition there was no change of collagen production with increasing mannitol concentrations (variations within 13% of basal values).

RESULTS

The (^3H)-proline incorporation (experimental counts per minute (CPM)/ μg DNA for the various glucose concentrations is expressed in per cent, standardized to 100% at the 1 mg/ml glucose level) into collagen in the medium is illustrated in Fig. 1. The figures are based on the pooled dishes divided into groups. At the various glucose levels (1, 3, 5, and 7 mg/ml medium, i.e. 5.6, 16.7, 27.8, and 38.9 mmol/l) we found no significant influence on the cells from type 2 diabetics. The fibroblasts from non-diabetic donors had an increased collagen production only at 5 mg/ml, which was highly significant, ($p < 0.001$). The cells from type 1 diabetics

Table 2. Results on individual donor basis.

The collagen production at various glucose concentrations in the medium presented at an individual donor basis. The donor number refers to the presentation in table 1. P = passage level in cell culture. At glucose 1 mg/ml is the absolute basal values denoted in counts per minute (CPM)/ μ g DNA ($M \pm S.E.M.$) and the 3H -proline incorporation into collagen is presented at 3, 5 and 7 mg/ml glucose concentrations as per cent values related to the basal. Numbers of dishes are presented in brackets.

Non-diabetics

Donor	P	Absolute basal values	Standardized values			
		(CPM)/ μ g DNA	1 mg/ml	3 mg/ml	5 mg/ml	7 mg/ml
1	5	7355 $\pm$ 145	100 (3)	61 (3)	124 (1)	77 (4)
2	5	1802 $\pm$ 98	100 (7)	107 (3)	151 (3)	149 (4)
2	5	1049 $\pm$ 86	100 (8)	65 (2)	153 (3)	128 (3)
3	5	9536 $\pm$ 337	100 (5)	---	123 (2)	---
3	7	5432 $\pm$ 198	100 (5)	107 (3)	100 (3)	98 (2)
4	9	3022 $\pm$ 235	100 (7)	104 (3)	114 (4)	---
5	3	5723 $\pm$ 366	100 (7)	99 (3)	108 (4)	---
6	4	4158 $\pm$ 11	100 (2)	74 (3)	79 (2)	83 (2)
7	7	5542 $\pm$ 628	100 (3)	98 (3)	122 (2)	88 (3)
8	7	1322 $\pm$ 91	100 (3)	161 (3)	126 (3)	121 (2)
MEAN			100 (50)	99 (26)	117 (27)	101 (20)
$\pm$ S.E.M.				10.5	7.3	10.1
Subjects			8	8	8	6

increased their collagen production into the medium significantly at the 3 and 5 mg/ml glucose levels compared to the basal (1 mg/ml). The increment was close to double at the 5 mg/ml glucose level. Accordingly, the collagen production was significantly different by cells from type 1 diabetics compared to cells from type 2 diabetic donors at glucose levels 3 and 5 mg/ml. No significant differences was found between cells from non-diabetics and type 2 diabetics at any glucose levels.

Similar results were obtained when calculating (Mann-Whitney test) on experimental results on individual donor subject basis. These data are presented in detail in table 2. It was confirmed that cells from type 1 diabetics displayed a significantly ($p < 0.001$) higher collagen production at glucose 5 mg/ml compared to 1 mg/ml. This was also a significant ($p < 0.01$) finding with cells from non-diabetics. No significant changes were found with cells

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(table 2 ctd)

Type 1 diabetics

Donor	P	Absolute basal values	Standardized values			
		(CPM)/ μ g DNA	1 mg/ml	3 mg/ml	5 mg/ml	7 mg/ml
9	3	1974 $\pm$ 309	100 (8)	80 (4)	---	77 (3)
10	3	641 $\pm$ 75	100 (7)	100 (3)	337 (4)	243 (3)
11	4	1672 $\pm$ 72	100 (4)	133 (2)	107 (3)	109 (2)
11	4	1847 $\pm$ 119	100 (2)	116 (4)	123 (4)	102 (3)
12	3	1765 $\pm$ 117	100 (5)	129 (3)	139 (2)	149 (3)
12	3	168 $\pm$ 21	100 (7)	288 (4)	255 (4)	331 (2)
13	8	441 $\pm$ 40	100 (8)	180 (4)	221 (3)	---
14	10	4410 $\pm$ 573	100 (7)	108 (3)	115 (3)	---
15	3	3032 $\pm$ 153	100 (3)	98 (3)	104 (3)	126 (2)
16	3	2132 $\pm$ 628	100 (3)	75 (2)	118 (3)	60 (3)
MEAN			100 (54)	121 (32)	175 (29)	139 (21)
$\pm$ S.E.M.				16.9	32.8	31
Subjects			8	8	7	6

Type 2 diabetics

17	5	388 $\pm$ 36	100 (4)	81 (2)	140 (3)	115 (4)
18	3	1302 $\pm$ 453	100 (6)	65 (3)	150 (4)	95 (2)
19	7	773 $\pm$ 54	100 (8)	99 (3)	96 (3)	95 (2)
20	7	5294 $\pm$ 151	100 (7)	102 (4)	134 (2)	---
20	2	5108 $\pm$ 582	100 (7)	136 (1)	115 (4)	128 (3)
21	3	872 $\pm$ 188	100 (8)	84 (4)	92 (4)	89 (2)
22	7	3529 $\pm$ 403	100 (6)	100 (4)	109 (4)	---
23	2	4057 $\pm$ 401	100 (5)	78 (2)	120 (3)	120 (3)
24	2	4639 $\pm$ 369	100 (6)	103 (3)	90 (3)	91 (4)
25	2	2500 $\pm$ 316	100 (8)	92 (2)	75 (4)	110 (2)
26	2	1135 $\pm$ 153	100 (2)	120 (2)	115 (2)	109 (3)
27	2	2220 $\pm$ 103	100 (2)	85 (3)	101 (3)	101 (3)
28	2	1730 $\pm$ 41	100 (2)	114 (3)	70 (2)	102 (2)
MEAN			100 (71)	94 (36)	107 (41)	105 (30)
$\pm$ S.E.M.				4.7	7.0	3.7
Subjects			12	12	12	11

from type 2 diabetics. A significant difference ($p < 0.05$) between type 1 and type 2 donors was only confirmed at the 5 mg/ml glucose concentration, on calculation on an individual donor basis. There were no significant differences at all regarding glucose influence on protein production, as mentioned above in "Statistics".

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No attempts have been made in this study to compare the different cell types at the basal level (1 mg/ml) because the cells were not grown simultaneously, nor were the experiments or analyses performed in parallel all over between the different cell lines.

The cell mass was intentionally different for the various cell lines. A careful seeding was performed, however, to keep the cell amount within a narrow range for all dishes at the start of the experiment in an individual cell line. There was no indication of a systematic change due to glucose influence on cell amount (DNA/dish). It is previously reported that collagen production per cell is independent of cell density (6), and in this study as well there was no relationship found between the glucose induced changes in collagen production and $\mu\text{g DNA/dish}$. Although various cell amounts contributes to the interexperimental variations it was decided important to evaluate the effect of glucose on collagen production at different cell densities.

The cells used in this study from type 1 diabetics (juvenile onset diabetes) and type 2 diabetics (maturity onset diabetes) were from subjects representative for each type of diabetes. Thus, there is a difference in donor ages between the two groups. There is no apparent age related tendency that could explain the findings described above.

The effect of glucose on protein production (Fig. 2) is generally much less pronounced than the increment noted in collagen production. A stimulatory effect was noted significantly (calculations based on pooled dishes) for all three types of cells at glucose 5 mg/ml.

The differences between the number of dishes in figures 1 and 2 are due to laboratory failures in analyses.

No significant differences between the cell types were found in glucose stimulation of total protein synthesis at any glucose level.

DISCUSSION.

The pathogenesis of diabetic macro- and microangiopathy remains to be elucidated, but is clearly multifactorial (7). Current available evidence favours the thesis that the complications are secondary to the metabolic disorder (8). In a recent report on a cross-sectional study on diabetic subjects interesting differences were noted in the risk factor profiles for clinical macrovascular disease. In the type 1 diabetic subjects there were no associated risk factors other than age, but in type 2 diabetics several independent risk factors were identified: age, creatinine, glucose, high-density lipoprotein cholesterol, total cholesterol, and systolic blood pressure (9). It is generally agreed that normalization of the glucose concentration in the blood is a primary aim in the treatment of diabetics. Nonenzymatic glycosylation of hemoglobin has become a widely accepted means (HbA_{1c}) for monitoring metabolic regulation. Subsequently, it has been shown that proteins other than hemoglobin also are subject to nonenzymatic glycosylation; these include serum proteins (albumin, α -2-macroglobulin and immunoglobulins), erythrocyte membrane proteins, basic myelin protein, actin, lens crystallin, and recently (10) also collagen. This is an important effect of glucose since several complications of diabetes mellitus occur in collagen-rich tissues.

To study the influence of glucose on collagen and protein production an in vitro system is suitable. Cultured human skin fibroblasts from diabetics may display a possible inherent defect (11, 12, 13, 14) in an otherwise controlled milieu. Villee and Powers (15) have demonstrated that fibroblasts, cultured from human subjects with non-metabolic disorders, growing in a high glucose medium secreted more collagen than those in glucose at a physiological concentration. They noted increasing amounts of collagen in the glucose concentration range 1-5 mg/ml, but above this range no further increase was noted. Their results are in essential agreement with our findings on cells from non-diabetic

donors, although we found no stimulatory effect of glucose 3 mg/ml. This difference may be due to experimental variations, such as the presence of serum in our study.

In this study collagen production by cells from type 1 diabetics was clearly dose-dependent at glucose concentrations 1-5 mg/ml. The absence of significant glucose effect on cells from type 2 diabetics is noteworthy. In essential, cells from type 2 diabetics secreted collagen and protein in a similar pattern as cells from non-diabetic donors.

The cells from type 1 diabetics secreted more collagen into the medium at all glucose levels above the basal concentration 1 mg/ml, thus differing markedly from the other cell types. In a report by Rowe et al (16) skin fibroblasts from JODM (type 1) diabetics were cultured and compared to fibroblasts from non-diabetic and AODM (type 2) subjects. They found that collagen and protein synthesis were independent of donor age. In our study there was likewise no correlation between age and relative increment of collagen production at 5 mg/ml glucose concentration (Table 2). Rowe et al also noted that fibroblasts from type 1 diabetics synthesized significantly more collagen and total protein per μ g DNA than did control cell strains. Type 2 cells did not differ from control cultures in the amount of collagen synthesized per cell. Finally, they observed a difference between the three cell types studied with regard to total protein and collagen synthesis in the presence of hydrocortisone in the medium. (The mechanisms for these observations were unclear.)

In the present study there is a difference between skin fibroblasts from type 1 diabetics and cells from non-diabetics and type 2 diabetics in glucose effect on collagen production. In fact, the study indicates that the incorporation of radioactive proline into collagen in the medium is influenced by glucose only in type 1 diabetic fibroblasts. The differences in glucose effects on collagen and protein production in this study are unexplained and merit further exploration. The results may be affected by differ-

ent rates of collagen catabolism in the medium, or by differences in amino-acid pool sizes or inherent control mechanisms operative in collagen and/or protein biosynthesis and secretion. These mechanisms remain to be explored. It is tempting, however, to suggest an inherent cellular defect. This possibility would be consistent with findings of increased production of heparan sulfate by skin fibroblasts from diabetics (type 1 and type 2) (17) and of differences between non-diabetic and diabetic skin fibroblasts in collagen production influenced by insulin (18). Fibroblasts from type 2 diabetics have also been found to deviate in collagen production when exposed to dextran sulfate (19).

It is clear that caution should be exercised in the extrapolation of observations from cell culture studies to the *in vivo* situation. However, it is of great interest to note the significant reduction in the width of skeletal-muscle capillary basement membranes obtained by improved blood glucose control in type 1 diabetic patients (20), in addition to earlier findings on blood glucose and atherosclerosis (1).

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The Effects of Glucose and Insulin on Beta-Hexosaminidase in Cultured Fibroblasts from Diabetic and Non-Diabetic Subjects

by

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SUMMARY

The effects of glucose and insulin in culture medium on the release and cellular content of beta-hexosaminidase were studied in fibroblast culture obtained from patients with type II diabetes and from control subjects. The release of beta-hexosaminidase was (positively) correlated to extracellular glucose concentration. The cellular activity was not influenced by glucose. Insulin had no effect on the cellular activity or the release of beta-hexosaminidase. The results were similar for both cell types. It is proposed that increased plasma levels of lysosomal hydrolases (e.g. beta-hexosaminidase) from human diabetics might be due to increased release of cells exposed to elevated glucose concentrations.

INTRODUCTION

Lysosomal acid hydrolases are involved in the intracellular digestion of endogenous and exogenous macromolecules. In culture certain cells (e.g. fibroblasts) release lysosomal hydrolases into the surrounding medium (1). Conditions analogous to cell culture may apply in vivo where lysosomal hydrolases are found in extracellular fluids in addition to their intracellular lysosomal location.

Previous studies have demonstrated increased activities of several lysosomal hydrolases (e.g. beta-hexosaminidase, beta-glucuronidase) in plasma from diabetic patients in close correlation with blood glucose levels (2-4).

In the present study we have therefore examined the effects of glucose and insulin on the intra- and extracellular activity of beta-hexosaminidase in cultured fibroblasts from non-diabetic and type II diabetic human subjects.

MATERIALS AND METHODS

Cell culture: Human fibroblasts were obtained from non-diabetic (control) subjects and diabetic patients. Controls had no cases of diabetes among first-degree relatives and had a normal oral glucose tolerance test. Diabetics were non-insulin dependent (type II), not prone to ketosis, and were on treatment with diet and, in two cases, low-dose sulphonylurea. Fibroblast cultures were established from 3 mm skin punch biopsies taken from the medial aspect of the left upper arm after informed consent. Ten to 20 explants were incubated in plastic flasks (NUNC A/S, Denmark) containing 2 ml minimal essential medium (Flow Laboratories, Scotland) with 20 mM HEPES and gentamicin 0.1 mg/ml, supplemented with 10% heat-inactivated fetal bovine serum, L-glutamine and non-essential amino acids. The flasks were incubated at 37°C and left untouched for 10 days to allow adherence and primary growth, which was usually observed within 2 to 3 weeks. The medium was changed twice a week. Subcultures were obtained by the use of 0.05% trypsin-0.02% EDTA. Cells from confluent cultures were trypsinised into experimental dishes and used as described. Cells were used in passages 2-8.

Experiments were performed in petri dishes (9 cm² NUNC A/S). After trypsinisation the cells were grown to confluence in the dishes. They were washed 3-5 times with phosphate-buffered saline before the start of experiments, which were performed in 3 ml glucose-free and sulphate-free medium (Flow Laboratories) with appropriate additions of glucose and insulin, but without fetal bovine serum. Pork monocomponent insulin (Actrapid^R, Novo, Denmark) was added to each dish at various concentrations. Glucose

was added from a 40% stock solution (ACO, Sweden). The cells were exposed to experimental conditions for 24 h. In all experiments fibroblast cultures from five diabetics and five controls were used.

Analytical techniques: The assay of beta-hexosaminidase was performed as described earlier (2) with p-nitrophenyl-beta-N-acetylglucosaminide (Koch-Licht, UK) as substrate. Enzyme assays were performed on fibroblasts scraped from the dishes and homogenised in 2 ml distilled water by an all-glass Potter-Elvehjem homogenizer. Incubation times at 37°C were 0, 30 and 60 min. Culture medium was analysed with an incubation time of 0, 6 and 18 h. One unit of enzyme activity represents 1 μ mol of substrate split per min. Protein was determined by the method of Lowry et al (5) and glucose by a glucose oxidase method.

For isoelectric focusing 2 ml of medium were applied to a 110 ml LKB column (LKB Products, Sweden) using ampholine with a pH range of 4-6. The samples were electrofocused for 42 h. The voltage and current at the end of the experiment were 550 V and 0.8 mA, respectively. Fractions of 2 ml were collected and of these, 100 μ l were incubated at 37°C for 120 min with 100 μ l of a 1 mmol/l solution of 4-methylumbelliferyl-beta-N-acetylglucosaminide (Koch-Light) dissolved in citrate-phosphate buffer pH 4.5. The amount of 4-methylumbelliferyl released was measured fluorometrically (1). Statistical differences were evaluated by the Mann-Whitney rank sum test. A P value less than 0.05 was considered significant.

RESULTS

The cellular activity and the release into medium of beta-hexosaminidase from fibroblasts obtained from type II diabetics and controls did not differ (Table I). Likewise, fibroblasts from these two sources responded identically to glucose and insulin as described below.

Various insulin concentrations (0, 0.1, 1, 10 and 100 mU/ml medium) tested at 0 and 5.6 mmol/l glucose exhibited no influence on the release or the cellular activity of beta-hexosaminidase. On the other hand, the release of beta-hexosaminidase (at 0 or 1 mU insulin per ml medium) was positively correlated to glucose concentration. Beta-hexosaminidase activity was significantly ($P<0.05$) increased in medium with glucose compared with medium with no glucose and activity in medium with more than 16.8 mmol/l glucose was significantly ($P<0.05$) higher than in medium with 5.6 mmol/l glucose (Table 2) However, the cellular activity was not influenced by glucose. The glucose consumption (measured as decrease in medium) was correlated to glucose concentration and was more than doubled in fibroblast cultures exposed to the highest

Table 1. Intracellular activity and release of beta-hexosaminidase (Median and range). Intracellular activity is given as U/g protein and the release as U/l medium. Insulin and glucose concentrations were 1.0 mU/ml medium and 5.6 mmol/l medium, respectively.

	Diabetic fibroblasts n = 5	Non-diabetic fibroblasts n = 5
Intracellular activity	51 (38-67)	49 (30-71)
Released activity	0.7 (0.5-0.9)	0.6 (0.4-0.9)

Table 2. Release of beta-hexosaminidase from five diabetic and five non-diabetic fibroblast cultures at different glucose concentrations. Activity released into medium without glucose is equal to 100%. Median and range are given. Insulin concentration in all experiments were 1.0 mU/ml medium.

Glucose concentrations (mmol/l)	Diabetic fibro- blasts (%)	Non-diabetic fibroblasts (%)
0	100	100
5.6	133 (124-158)	128 (120-148)
16.8	156 (134-167)	150 (131-220)
39.2	190 (151-256)	182 (144-234)

glucose concentrations (i.e. 33.6 and 39.2 mmol/l medium) compared to those with 5.6 mmol glucose (Table 3).

To evaluate the possibility of an osmotic effect on the enzyme release, mannitol was added at the same concentrations as glucose. However, no influence was noticed on the intracellular content or release of beta-hexosaminidase.

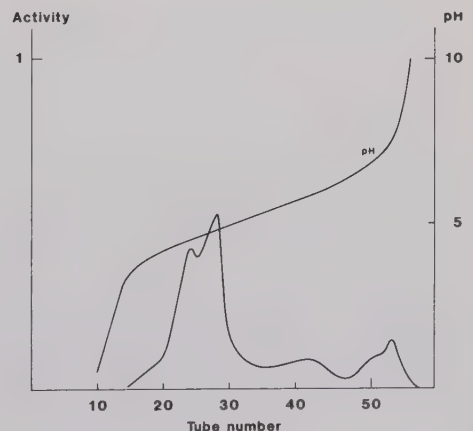
Isoelectric focusing of the released beta-hexosaminidase in media (Fig 1) with different glucose concentrations did not reveal

Table 3. Glucose consumption at 5.6 mmol/l and 33.6-39.2 mmol/l of glucose in medium (median and range). Glucose consumption is given as mmol/l).

	5.6 mmol/l (n = 4)	33.6-39.2 mmol/l (n = 8)
Diabetic fibroblasts		
with insulin (1 mU/ml)	0.7 (0.6-0.8)	1.7 (1.1-2.5)*
Diabetic fibroblasts		
without insulin	0.6 (0.4-0.7)	1.5 (0.9-2.8)*
Non-diabetic fibroblasts		
with insulin (1 mU/ml)	0.7 (0.6-0.8)	1.9 (1.2-3.3)*
Non-diabetic fibroblasts		
without insulin	0.5 (0.4-0.7)	1.6 (1.4-1.8)*

P<0.05 compared with values at 5.6 mmol glucose/l (Mann-Whitney rank sum test).

Figure 1. Isoelectric focusing of beta-hexosaminidase from 2 ml of medium as described in "Materials and methods". Activity is given as nmol substrate split per hour.



any change in the isoenzyme pattern compared to earlier described pattern (1).

DISCUSSION

In several studies fibroblasts derived from non-diabetics and diabetics have been used to investigate mechanisms related to the pathophysiology of the diabetic state. These studies have for instance given conflicting results with respect to glucose transport and metabolic effects of insulin on cells from diabetics compared with non-diabetics (6-7). In this study we found no difference in the intracellular activity or the release into medium of beta-hexosaminidase in cells from diabetics and controls. Likewise, the response to insulin or glucose in these respects was identical.

In diabetes, there is evidence relating the vascular complications to increased thickness of the basal membrane (8). It is not clear, however, whether the presence of excess collagen-like glycoprotein material in the membrane is a result of deposition alone or a combination of this and impaired removal. Lysosomal hydrolases (e.g. beta-hexosaminidase), involved in glycoprotein catabolism, have been implicated in the pathogenesis of microangiopathy (3, 4, 9-11). Previous studies have shown a positive correlation between lysosomal hydrolases in serum and blood glucose (2-4) or retinopathy (3, 4, 9). In this study the release of beta-hexosaminidase from cultured diabetic and non-diabetic fibroblasts was positively correlated to glucose concentration in medium. The increased enzyme release was apparently not due to osmotic changes as the presence of mannitol in culture medium did not influence the enzyme release.

Insulin did not affect enzyme release or intracellular activity of beta-hexosaminidase in these short-term experiments. It is known, however, that rat fibroblasts cultured for 6 days in an insulin-enriched medium show significantly reduced release of

beta-hexosaminidase, whereas the intracellular activity is not influenced (12).

We propose that increased levels of lysosomal hydrolases in the plasma of human diabetics might be due to increased release from cells exposed to elevated glucose concentrations in vivo. Possibly the effect may be related to cellular metabolic changes induced by the increased glucose consumption observed in cells exposed to high extracellular glucose concentrations.

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